To the Graduate Council:

I am submitting herewith a dissertation written by Mary Crecink Kot entitled "Spermatogenesis in XOSxr, XXSxr, and XYSxr Mice." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

Mary Ann Handel

Mary Ann Handel, Major Professor

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and recommend its acceptance:

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SPERMATOGENESIS IN XOSxr, XXSxr, AND XYSxr MICE

A Dissertation

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ABSTRACT

The focus of this study was the investigation of the role of the Y chromosome in spermatogenesis by the analysis of morphological and biochemical aspects of spermatogenesis in XX*Sxr* males, in XY*Sxr* males, and, in particular, in XO*Sxr* males, which lack a significant portion of the Y chromosome, but have a single X chromosome, as do normal XY mice. The results suggest that a gene controlling Sertoli cell differentiation is encoded within the *Sxr* segment. In addition, it was found that meiotic cells, as well as post-meiotic cells, were significantly reduced in number in these mice. The reductions in spermatocyte and early spermatid numbers are probably due to improper sex chromsome pairing and/or inactivation. The dramatic reduction in spermatid number may also be due to a decrease in the communication between spermiogenic cells and Sertoli cells. All spermatocyte-specific and spermatid-specific structures, and at least two spermatid-specific proteins (basic nuclear proteins and LDH-C_4), were assembled in the correct temporal and spatial pattern in XO*Sxr* mice. These observations suggest that these structures are not encoded on the large portion of the Y chromosome that is missing in XO*Sxr* mice. Morphological abnormalities observed in pachytene spermatocytes and in spermatids were not unique to mice carrying the *Sxr* mutation and may reflect a disturbed testicular environment rather than the lack of Y-encoded products. Studies assessing sperm function demonstrated that sperm from XO*Sxr* mice could bind to the zonae pellucidae of mouse eggs, although they did so at a reduced efficiency. These data

indicate that the receptors necessary for sperm binding to the zona pellucida are present and, therefore, the genes which encode them must not be part of the Y chromosome missing in XO<u>Sxr</u> mice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The purpose of this study was to evaluate the genetic role of the Y chromosome in spermatogenesis by assessing spermatogenesis occuring in the absence of most of the Y chromosome. The experimental model was XOSxr mice. Differences in kinetic and morphological characteristics of spermatogenic cells between XOSxr males and normal males can be attributed directly or indirectly to Y-chromosome function.

Spermatogenesis in the Mouse

Spermatogenesis is a complex series of events involving three phases: mitotic proliferation of stem cells, meiosis, and the differentiation of haploid spermatids, or spermiogenesis. Stem cell spermatogonia undergo a precise series of mitoses and differentiate as the various spermatogonial cell types before entering pre-meiotic DNA synthesis. Pre-leptotene spermatocytes then enter an extended period of prophase. During prophase I the chromatin undergoes condensation (during leptotene), homologous chromosomes pair (during zygotene), and chromosomal segments are exchanged between nonsister chromatids and extensive RNA synthesis occurs (during pachytene). Early in prophase I, spermatocytes move away from the basement membrane of the seminiferous tubule and into the specialized microenvironment of the adluminal compartment of the tubule created by the Sertoli cell junctional complexes. Meiosis I culminates in the segregation, or

disjunction, of homologous chromosomes and the formation of two secondary spermatocytes. This division is usually called a reduction division since the number of separate centromeres is reduced to half in the two resulting daughter cells. After a short interphase in which no chromosomal DNA is replicated, meiosis II ensues and results in the production of haploid spermatids. The spermatids enter an elaborate process of cytodifferentiation, called spermiogenesis, during which sperm-specific structures such as the acrosome, sperm tail, manchette, and condensed and elongated nucleus, are differentiated. During early spermiogenesis, the acrosome and sperm tail are formed. The acrosome, derived from the Golgi complex, will eventually cover about one-third to one-half of the nuclear surface and contains the enzymes necessary for the sperm to penetrate the investments of the egg. The tail is initially an axonemal complex but will ultimately include nine outer dense fibers and a fibrous sheath, as well. The early events of acrosome formation and tail initiation are followed by elongation and condensation of the nucleus and elaboration of the manchette, a microtubular array enclosing the nucleus and is thought to be important in sperm-head shaping. The sperm head will eventually be flattened and fusiform in shape. The differentiated spermatids are released into the tubular lumen and transported to the epididymis where further maturation occurs.

While these processes of spermatogenesis have been structurally characterized, very little is known about the genetic mechanisms that control them. In particular, it is not known whether the Y chromosome plays a role in this complex process of cytodifferentiation.

Role of the Y Chromosome in Spermatogenesis

The Y chromosome has at least one essential function in mammals, that of primary sex determination. Studies of mice and humans with sex-chromosome aneuploidies have demonstrated that the Y chromosome is essential for male sex differentiation and that when it is present, as in XY and XXY individuals, embryos develop as males regardless of the number of X chromosomes (Jacobs and Strong, 1959; Russell and Chu, 1961). However, in the absence of the Y chromosome, as in XX and XO individuals, embryos develop as females (Ford et al., 1959; Welshons and Russell, 1959). These observations demonstrate that the Y chromosome is responsible for male sex determination in mammals.

In contrast, evidence for a role of the Y chromosome in spermatogenesis is limited (reviewed by Handel, 1987). The available information is incomplete in its scope and often based on inferences. Nevertheless, some experimental results suggest that the Y chromosome is involved in the control of spermatogenesis. For example, Levy and Burgoyne (1986) have used observations of an XO <—> XY male chimera to suggest that the Y chromosome is necessary for spermatogenesis. Even though this chimera appeared to be composed predominantly of XO cells (inferred from coat color pattern and an analysis of isozymes of

somatic tissues), spermatogenesis occurred only in XY cells, not in XO cells. Chromosomal analyses of spermatogenic cells revealed that XO germ cells exhibited early spermatogenic failure and did not contribute to the meiotic cell population. Therefore, it was concluded that there is a spermatogenesis gene, Spy, on the Y chromosome. However, this work did not provide specific evidence for a single gene. Further evidence for the role of the Y chromosome in spermatogenesis has come from the work of Burgoyne et al. (1986), who found evidence that a specific region of the Y chromosome is necessary for the onset of spermatogenesis. This evidence came from the comparison of two types of sex-reversed male mice that lack most of the Y chromosome; one type, the "common" sex-reversed (XOSxr) male mouse was positive for the male histocompatibility antigen (H-Y), while the other lacked H-Y (XOSxr') and presumably had suffered a small chromosomal deletion. Although all stages of spermatogenic cells were found in XOSxr males, the seminiferous tubules of XOSxr', H-Y-negative males were largely devoid of germ cells and contained only Sertoli cells. Analyses of neonatal mice suggested a failure in the early differentiation and proliferation of spermatogonia in these males. Therefore, these data suggested that a Y-linked gene, possibly that controlling H-Y antigen, Hya, acts early in testis development and the onset of spermatogenesis. Whether or not this gene is identical to the Spy gene is unknown.

Other evidence for Y-linked factors involved in spermatogenesis has come from the analysis of sperm morphology in certain congenic lines of mice. Krzanowska (1971, 1976) investigated the inheritance of sperm-head abnormalities by creating congenic lines of mice which differed only in the origin of their Y chromosome. The original parental strains were characterized by different percentages of sperm-head abnormalities. The resulting congenic mice carried the Y chromosome derived from the strains with low percentages of abnormal sperm; while the autosomes of the congenic line were derived from strains with high percentages of sperm abnormalities. The congenic mice had percentages of abnormal sperm which were lower than the parental strain contributing the autosomes, but higher than the parental strain contributing the Y chromosome. These data suggested that the Y chromosome, at least in part, determined the percentages of abnormal spermatozoa found in a given line. In addition, these data suggested that autosomal or X-chromosomal factors were also involved in the determination of sperm head parameters.

Further evidence for a role of the Y chromosome in sperm morphology derived from studies of Styrna et al. (1989). They utilized two congenic lines of mice; one line carried a variant Y chromosome presumed to have a small deletion and the other line carried a normal Y chromosome. The congenic line with the "partially deleted" Y chromosome was characterized by a high percentage of sperm-head abnormalities whereas the congenic line with a cytologically normal Y chromosome had a lower percentage of abnormal sperm. Matings

were made from both congenic lines to females from a number of inbred strains, and sperm from the resulting F_1 hybrids were examined. Normally, a decrease in the number of sperm abnormalities (a heterosis effect) would be expected to occur in the F_1 generation. However, Styrna et al. (1989) did not find a significant heterosis effect when the male parent in the mating carried the deleted Y chromosome. In contrast, when the male parent carried the normal Y chromosome, Styrna et al. (1989) found, as expected, a heterosis effect (a decrease in sperm abnormalities) relative to the paternal strain. These data suggested that factors on the Y chromosome, which were absent in the mutant line with the variant Y chromosome but present in the normal congenic line, have an influence on the percentage and production of normal spermatozoa.

Although the evidence reviewed here suggests that the Y chromosome may affect spermatogenesis, the loci, products, and modes of action of genes controlling the spermatogenic processes, either directly or indirectly, are largely unknown. Therefore, the existence of such genes on the Y chromosome must still be questioned. At least three possibilities concerning its role in spermatogenesis exist. First, the mammalian Y chromosome may contain genes responsible for the structures and controlling elements needed to produce functional spermatozoa. However, no such genes have been positively identified, possibly because appropriate experiments have not been designed. Second, it is conceivable that the Y chromosome contains only sequences necessary for the initial activation of a cascade of

spermatogenic genes located elsewhere, either on the X chromosome or on the autosomes. Third, it is possible that the Y chromosome contains no spermatogenic genes per se but functions simply as a pairing partner for the X chromosome during spermatogenesis, thereby assuring completion of meiosis. Several cases of sex-chromosome pairing failure leading to spermatogenic failure have been reported. For example, XYY mice are characterized by meiotic and post-meiotic abnormalities in spermatogenic cells suggesting that two Y chromosomes may somehow interfere with the normal synapsis of the sex chromosomes (Burgoyne and Biddle, 1980). In addition, mice with high levels of early X-Y disjuction display spermatogenic failure (Beechey, 1973; Imai et al., 1981; Matsuda et al., 1982, 1983). These cases suggest that normal X-Y pairing is necessary for the survival of meiotic and post-meiotic germ cells.

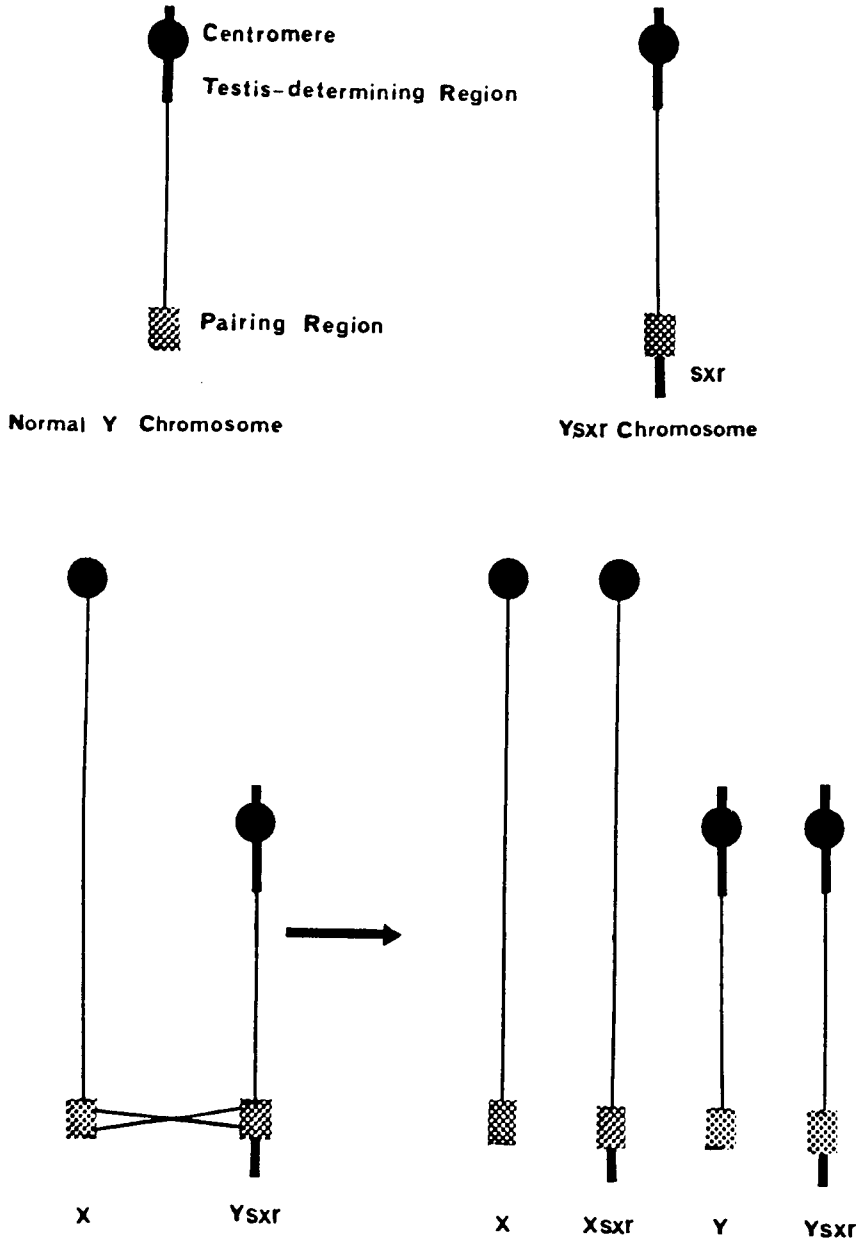
In summary, although the Y chromosome may indeed influence spermatogenesis, definitive genetic evidence for such a role is lacking. It is not known if the role of the Y chromosome in spermatogenesis is direct or indirect. Further, it is not known if there are any genes on the Y chromosome which control the production or assembly of sperm-specific structures or functions. It has been difficult to provide a genetic resolution of these issues since there are no well defined variants of the murine Y chromosome.

One approach for investigating the role of the Y chromosome in spermatogenesis is to study spermatogenic events in the absence of the Y chromosome. This approach was successful for studying the role of

the Drosophila Y chromosome in male fertility and was possible because in Drosophila an X-autosome ratio is sex-determining and the Y chromosome is not. A ratio of two X chromosomes to two sets of autosomes determines female development whereas, a ratio of one X chromosome to two sets of autosomes determines male differentiation. Thus, XO individuals are male. Since these males exhibit abnormal spermatogenesis, their analysis has provided genetic evidence for fertility factors on the Drosophila Y chromosome. However, since the Y chromosome is essential for normal male sex determination and testis formation in mammals, it is not possible to study spermatogenesis in the complete absence of the Y chromosome.

An alternative approach in mammals is to investigate spermatogenesis in the absence of most of the Y chromosome utilizing the sex-reversed (Sxr) mutation, more appropriately termed Tp(Y)1Ct. The Sxr region is a duplication of the portion of the Y chromosome that includes the sex-determining sequences. The duplicated sequences were transposed to the distal end of the Y in the original mutant males (Evans et al., 1982; Singh and Jones, 1982; Fig. 1). During meiosis in XYSxr males the Sxr region can be transferred from the Y to the X chromosome by a cross-over event (Evans et al., 1982; Fig. 1). Thus, Sxr is inherited in an apparently "pseudo-autosomal" manner with one half of the XX progeny and one half of the XY progeny of XYSxr males receiving the Sxr factor (Cattanach, 1975; Cattanach et al., 1971). XX and XO individuals inheriting the Sxr factor on their paternally derived X chromosome develop as phenotypic males (McLaren,

Figure 1. Diagram of the Hypothesized Origin of Sxr.



Mating with XX female produces XX XXSxr XY XYSxr

Mating with XO female produces XX XXSxr XY XYSxr
XO XOSxr

1983). Therefore, three types of male mice can carry the Sxr factor: XYSxr, XXSxr, and XOSxr. Each of these genotypes produces a characteristic phenotype. XYSxr mice are fertile males that exhibit a mosaic pattern of normal and abnormal spermatogenesis within their seminiferous tubules (Hannappel and Drews, 1979). XXSxr mice are phenotypic males that are sterile and are characterized by a complete lack of germ cells in adult testes (Cattanach et al., 1971). XOSxr mice are also phenotypic males and sterile, but they differ from XXSxr mice in that they have germ cells which complete spermatogenesis (Cattanach et al., 1971). Since spermatogenesis is apparently abnormal in XOSxr mice, they may provide clues about the role of the Y chromosome in spermatogenesis.

The focus of this study was the analysis of spermatogenic kinetics and differentiation, as well as sperm function, in XOSxr males that lack most of the Y chromosome. Germ cells of these mice have been compared to those of normal XY and Sex-reversed-bearing mice, both XYSxr and XXSxr. Analysis of XYSxr, XXSxr, and XOSxr males can be expected to provide insights into the nature of Sxr and the role of the sex chromosomes in male germ-cell development.

XYSxr mice are fertile carriers of the Sxr factor (Cattanach et al., 1971). All XYSxr mice have testes that are reduced in weight (approximately one half that of normal XY littermates) due to a decrease in seminiferous tubule diameter (Cattanach et al., 1971; Hannappel and Drews, 1979). Cattanach et al. (1971) also found a high percentage of abnormal sperm in XYSxr mice. Although germ-cell

reduction may be responsible for these phenotypes, previous studies have not provided accurate quantification of germ-cell numbers in these males. Hannappel and Drews (1979) reported that seminiferous tubules of XY*Sxr* mice contained patches of normal and abnormal spermatogenesis. Large individual variations in the number of abnormal cells among XY*Sxr* mice were found. However, interpretation of these data is difficult since not all cell types and stages of the seminiferous epithelial cycle were examined in this study. Meiotic analyses indicated that XY*Sxr* mice exhibited a higher than normal frequency of X-Y univalence at diakinesis-metaphase I, varying from 70-90% (5-10% in normal XY littermates) (Winsor *et al.*, 1978; Chandley and Fletcher, 1980; Evans *et al.*, 1980). Lyon *et al.* (1981) suggested that this abnormal association of the sex chromosomes was due to a separation at the first metaphase division. However, Chandley and Speed (1987) observed that the disturbance first occurred during prophase I and was perhaps due to the self-pairing of the Y chromosome. In order to explain abnormalities in spermatogenesis in XY*Sxr* mice, Hannappel and Drews (1979) postulated that abnormal spermatogenesis resulted from the loss of the Y chromosome due to improper pairing, and the data of Chandley and Speed (1987) supported this suggestion.

The second type of mice carrying the *Sxr* factor are XX*Sxr* males. These individuals carry the *Sxr* region on the paternally derived X. Although XX*Sxr* males can mate and produce vaginal plugs, they are sterile (Cattanach *et al.*, 1971). The seminiferous tubules are

reduced in size and completely devoid of germ cells in the adult (Cattanach, 1975), even though at 16 days of fetal development germ cells are present within the testis (Cattanach, 1975). Most of these germ cells follow the typical male progression and enter mitotic arrest before birth (13-15 days post coitum; McLaren and Monk, 1981). However, XXSxr cells apparently degenerate soon after birth or, in some cases, during fetal development (Cattanach et al., 1971). This degeneration may be due to the presence of two X chromosomes which appears, in some way, to be detrimental to germ cells within the testis. A similar situation occurs in XXY mice which also exhibit early germ-cell degeneration (Handel and Hunt, 1989). A few of the XXSxr germ cells apparently follow a female path of differentiation and enter prophase of meiosis I before birth, and subsequently, degenerate (McLaren, 1981). These fetal germ cells observed in meiosis were usually located near the mesonephric rete region which has been hypothesized to produce a meiosis-inducing substance (Byskov and Saxen, 1976).

XOSxr individuals are the third category of mice carrying the Sxr factor, and were the focus of this study. These mice are phenotypic males with active spermatogenesis; however, they are sterile (Cattanach et al., 1971). Nonetheless, they are able to copulate with females, suggesting near-normal male endocrine differentiation and function (Cattanach et al., 1971). They also express H-Y antigen which supports the hypothesis that the Sxr factor contains the gene controlling H-Y antigen (Koo, Reidy, Nagamine, 1983). The testes of

XOSxr mice are larger than the testes of XXSxr males, but smaller than testes from XYSxr or XY males (Cattanach, 1975). Until the present study, little was known about the kinetics or morphology of spermatogenic cells in XOSxr mice. It was not known what percentage of germ cells do complete meiosis and progress through the various stages of spermiogenesis. Although Cattanach et al. (1971) reported that spermatogonia and spermatocytes appeared normal, no specific morphological characterizations, numbers, or tubule stages were included in his study. In addition, it had been reported that some germ-cell loss occurred among pachytene spermatocytes (Burgoyne et al., 1985).

The morphological information about spermatids from XOSxr mice is limited and has often been based on unreliable samples. For example, abnormal areas of seminiferous tubules have been reported from both XXSxr and XYSxr mice containing cells that seem to resemble spermatids of XOSxr mice (Hannappel and Drews, 1979; Burgoyne and Baker, 1984). Reduction in the numbers of spermatids in XOSxr mice has been reported (Cattanach et al., 1971) but was not based on quantitative and statistical analyses. In addition, XOSxr mice, as well as XYSxr mice, appear to have two classes of round spermatids: large round spermatids and normal-sized round spermatids (Hannappel and Drews, 1979; Hannappel, et al., 1980; Burgoyne and Baker, 1984). However, there has not been a quantitative analysis of these classes. Furthermore, the presence and morphology of sperm-specific structures in XOSxr spermatids has not been addressed. This is an important

consideration, since the absence of such structures could indicate a role for the Y chromosome in specifying these sperm-specific structures. Conversely, the presence of these structures in spermatids from XOSxr males would indicate that the genes encoding sperm-specific structures are not located within the portion of the Y chromosome that is missing in XOSxr mice.

Hannappel et al. (1980), Levy and Burgoyne (1986), and Burgoyne (1987) analyzed the DNA content of round spermatids from testicular suspensions from 16 XOSxr males by Feulgen microdensitometry. Burgoyne and his collaborators found that 35-94% of these round spermatids have the diploid amount of DNA. Levy and Burgoyne (1986) felt that the presence of diploid spermatids in XOSxr mice was not a direct effect of the Sxr factor but was a more general manifestation of disturbed spermatogenesis and meiosis. Thus, spermatogenesis is not normal in XOSxr mice; although spermatozoa are formed, they are non-motile and morphologically abnormal. However, these data were based on qualitative observations (Cattanach, 1975). The types, frequencies, and ultrastructure of sperm abnormalities are unknown. In addition, while it is known that XOSxr are infertile in vivo, it is not known whether the mature spermatozoa can accomplish fertilization in vitro. More specifically, the ability of sperm from XOSxr mice to function in any aspect of fertilization, such as binding to the zona pellucida or activation of the egg, is unknown.

XOSxr Mice as a Model for Investigating the Role of the Y Chromosome in Spermatogenesis

XOSxr mice provide an excellent model for studying the role of the Y chromosome in spermatogenesis. There is only a small portion of the Y chromosome present in XOSxr mice. Furthermore, these mice have one sex chromosome. Unlike XXSxr mice, XOSxr mice do not have a second X chromosome that could interfere with spermatogenic function. These aspects make it possible to assess the function of the genetic material within the portion of the Y chromosome that is missing in XOSxr mice. Finally, it is possible with XOSxr mice to determine if factors related to the normal behavior of the sex chromosomes are important in the control of spermatogenesis.

Since XOSxr mice lack a significant portion of the Y chromosome, yet are able to support spermiogenic differentiation, a number of experimental questions concerning the role of the Y chromosome in spermatogenesis were addressed in this study. First, do spermatids of these mice lack any specific structures that might be encoded or controlled by a gene or genes on the portion of the Y chromosome that is missing in XOSxr mice? Second, is spermatogenesis quantitatively normal in XOSxr mice, and if not, when in the course of spermatogenesis do disruptions occur? Does the time at which abnormalities first occur provide any clues as to the genic or chromosomal function of the Y chromosome? Third, are the spermatozoa of XOSxr mice morphologically normal and, if not, is there any evidence for sperm-morphology genes on the Y chromosome? Fourth, do

spermatozoa function in various aspects of fertilization? Might there be genes on the Y chromosome that control these functions? Although the Y chromosome may have a role in spermatogenesis, the extent and mechanisms of the contribution of the Y chromosome to sperm differentiation have previously been unknown. XO<u>Sxr</u> mice, lacking a major portion of the Y chromosome, provide a logical model for experimental resolution of some of these problems.

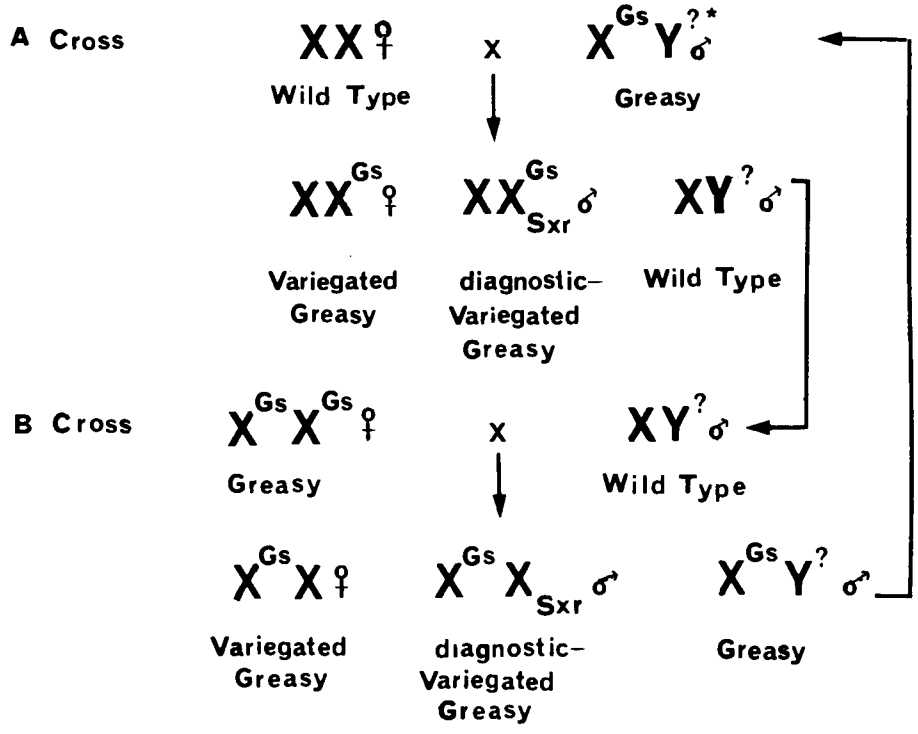
II. MATERIALS and METHODS

Animals

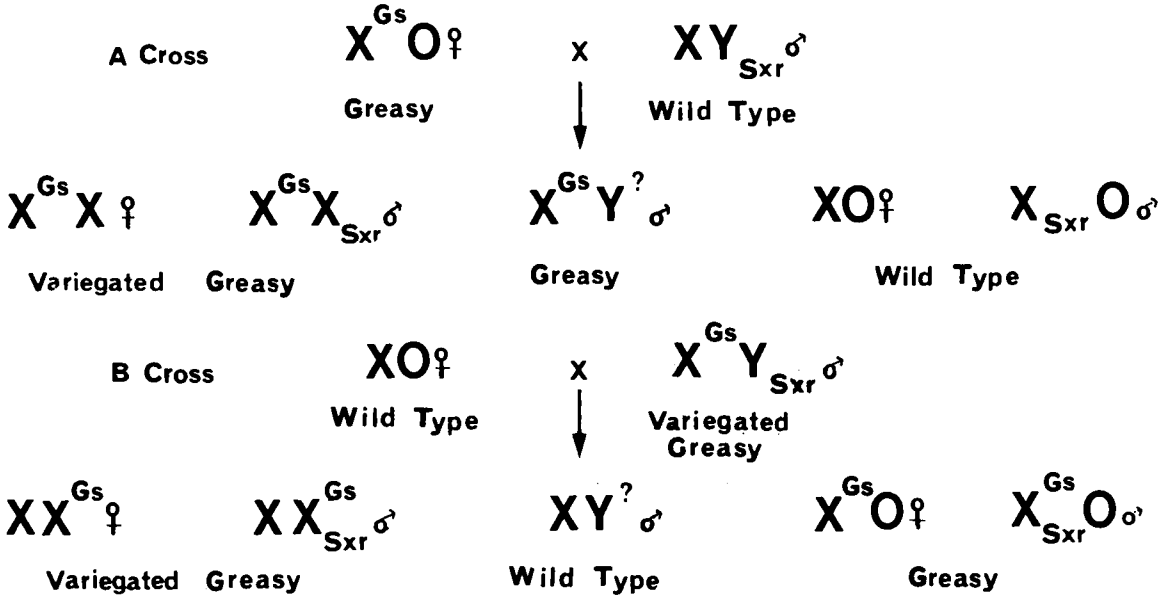
XO*Sxr* mice were obtained from matings between XY*Sxr* males and XO females. The XY*Sxr* males and XO females were either genetically unmarked or hemizygous for one of the two sex-linked markers, Tabby (*Ta*) or Greasy (*Gs*). Marked XY*Sxr* mice (hemizygous for *Ta* or *Gs*) were mated to X^{+}/O females, whereas unmarked XY*Sxr* mice were mated to $X^{\underline{Gs}}/O$ or $X^{\underline{Ta}}/O$ females. The sex-linked markers were used to determine the genotype from the coat phenotype of the offspring. (See Fig.2 for a diagram of the mating scheme.) XY*Sxr*, XX*Sxr*, and XY^{+} littermates served as controls to the XO*Sxr* mice. In addition, when these littermate controls were not available, normal HA/ICR, CD-1, and Balb/c males were used. XY*Sxr* mice were initially obtained from the Oak Ridge National Laboratory or the Jackson Laboratory; XO females were obtained from the Jackson Laboratory; and HA/ICR, CD-1, and Balb/c strains were obtained from Charles River Breeders (Wilmington, MA). XO*Sxr* mice used in this study ranged in age from 3 to 10 months with a mean age of 6.2 months; XY*Sxr* mice ranged in age from 8 to 23 months, with a mean age of 14.6 months; and XY mice ranged in age from 3 to 8 months, with a mean age of 4.9 months. Older XY*Sxr* mice were used out of necessity, since younger XY*Sxr* mice were needed for breeding purposes. Animals were maintained on a 14-hour light:10-hour dark schedule with lights on at 5 a.m. Food and water were given *ad libitum*.

Figure 2. Mating Schemes Used to Generate XY<u>Sxr</u> and XO<u>Sxr</u> Mice.

Mating Scheme Used to Generate XYSxr Mice



Mating Schemes Used to Generate XOSxr Mice



*Y? indicates an undefined genotype and may be either Y+ or YSxr

Ta may be substituted for Gs in the mating schemes.

Light Microscopy

Mice were killed by cervical dislocation. Testes were removed, fixed in Bouin's or Zenker's fixative, dehydrated, and embedded in plastic (glycol methacrylate) or paraffin. Blocks were sectioned at 3 to 5μm, mounted on albumin-coated slides, and stained with periodic-acid Schiff reagent (PAS) and hematoxylin. The stage of twenty-four, round seminiferous tubules per testis per animal was determined according to the criteria described by Oakberg (1956).

Considerable attention was given to morphological staging of spermatogonia and prophase spermatocytes. Criteria for staging of Type A spermatogonia were based on those of Oakberg (1971). Stem cells are pale with homogeneously grainy nuclei and, like all spermatogonia, are located at the base of the seminiferous tubule. The morphology of Type A spermatogonia varies with the stage of the seminiferous epithelium (Oakberg, 1971), but these cells have generally oval nuclei with partially condensed chromatin, depending on the stage, few nucleoli, and flakes of heterochromatin located adjacent to the nuclear envelope. Intermediate spermatogonia have oval nuclei with clumps of heterochromatin located adjacent to the nuclear envelope, although centrally located heterochromatin may be present in late Intermediate spermatogonia. Type B spermatogonia have oval to round nuclei and prominent, deeply staining clumps of heterochromatin located throughout the nucleus. The presence of central heterochromatin distinguishes these cells from Type A spermatogonia. Nuclei of early pre-leptotene spermatocytes are

smaller than those of Type B spermatogonia. Initially, the chromatin of these cells resembles that of Type B spermatogonia but they accumulate evenly dispersed, small heterochromatic clumps. Leptotene spermatocytes are basally located in the tubule, have small nuclei with evenly dispersed, densely staining, grainy chromatin. These heterochromatic clumps become globular, giving the nuclei a raspberry-like appearance. The chromatin of the round nuclei of zygotene spermatocytes is compact and highly heterochromatic. During pachynema, the nuclei enlarge and the sex vesicle becomes prominent by the mid-pachytene stage. The chromatin is visible in rope-like strands that become thin and diffuse. Spermatocytes in the diplotene stage have highly extended, fuzzy ribbons of chromatin and the sex vesicle often appears detached from the nucleus. Cells in the diakinesis stage are recognized by the clumps of chromatin with a doughnut-like appearance, representing the contracted chromosome pairs.

A Bioquant I Image Analysis System was used to analyze size classes of round spermatids from photomicrographs. Two hundred cells per step of spermatid differentiation were measured for each genotype (XY^{+}, XY<u>Sxr</u>, and XO<u>Sxr</u>). Ten electron micrographs of pachytene spermatocytes from each animal were used for measurements of the sex vesicle.

For identification of nuclear proteins, testes were fixed in Bouin's solution, sectioned at 6.5 to 7μm, mounted on albumin-coated slides, incubated in 5% trichloroacetic acid (TCA) at 60°C for 3 hours

to hydrolyze the DNA, washed in ethanol and phosphate buffer (pH 7.0), and stained with either 1.0% Fast Green or 0.1% Brilliant Sulfaflavin in phosphate buffer (pH 7.0). Control slides were treated with a 5% sodium nitrite and 5% acetic acid solution before DNA hydrolysis to block staining of lysine residues. Sections stained with Fast Green were examined using a Zeiss light microscope; sections stained with Brilliant Sulfaflavin were examined using a Leitz video-intensification microscope equipped with epifluorescence. Kodak Ektachrome 35mm color film (ASA 800) was used to photograph the tubules. These methods were a modification of those described by Hauschteck-Jungen and Hartl (1975).

Methods of Hintz and Goldberg (1972) were used for detection by indirect immunofluorescence of the sperm-specific isozyme of lactate dehydrogenase-C_4 (LDH-C_4) in testes fixed in Bouin's solution and paraffin-embedded. A Leitz microscope equipped with epifluorescence was used. Kodak Ektachrome 35mm color film (ASA 800) was used for photography.

Epididymal sperm were examined and scored for morphological abnormalities following the procedure and criteria of Kot and Handel (1987).

Electron Microscopy

Mice were anesthetized with Metofane (methoxyflurane inhalant) and perfused by inserting a canula through a small cut in the left ventricle. A buffered solution containing 0.8% sodium chloride, 0.4%

dextrose, 0.8% sucrose, 0.067M sodium cacodylate and 0.001M Mg^{++} and Ca^{++} (chloride salts) was perfused through the heart, followed by perfusion with 100ml of primary fixative (2.0% paraformaldehyde, 3.0% glutaraldehyde, 4.0% sucrose, 0.067M sodium cacodylate, 0.001M Mg^{++} and Ca^{++}, pH 7.35) and then with 100ml of the second fixative (4% paraformaldehyde, 6.0% glutaraldehyde, 4.0% sucrose, 0.067M sodium cacodylate, 0.001M Mg^{++} and Ca^{++}, pH 7.2). Testes were removed, weighed, and fixed for an additional hour in the second fixative. The tissue was then washed twice in 0.1M sodium cacodylate buffer with 0.001M Mg^{++} and Ca^{++} and stored at 4°C overnight. The testes were post-fixed in cacodylate-buffered 1% osmium tetroxide, dehydrated through ethanols, and embedded in Epon. Thin sections were cut with a Reichert OMU-2 ultramicrotome, staining with lead citrate and uranyl acetate, and examined with a Zeiss 98 or Hitachi H-600 electron microscope.

Autoradiography of Transcription

Incorporation of [^{3}H]-uridine into nuclei of pachytene spermatocytes was used to determine the transcriptional activity of the sex chromosomes. The method was adapted by Handel and Park from those of Wessels-Kaalen et al. (1986). Mice were killed by cervical dislocation. The testes were removed and de-tunicated, then minced in 2ml of Eagle's Minimum Essential Medium (MEM). [^{3}H]-uridine (300μCi) was added to the medium and the cell suspension was incubated at 33°C in 5% CO_2 in air. After 1 hour the cells were centrifuged, washed two

times in cold 2.2% sodium citrate and two times in 1.0% sodium citrate, fixed in 3:1 methanol:acetic acid for 1 hour, centrifuged, and resuspended in the same fixative. The cell suspension was dropped onto pre-cleaned slides warmed to 37°C and placed at a 10° angle. The preparations were allowed to dry overnight, then dipped in Kodak NTB2 emulsion, incubated at 4°C for 24 hours, developed in D-19 developer, fixed in Kodafix, and stained in Gurr Giemsa and phosphate buffer (pH 6.8; 1:15). The number of silver grains over each pachytene spermatocyte nucleus and sex vesicle was determined, as well as a background grain count for each cell. Grain counts over the entire nucleus minus the background, in addition to grain counts over the sex vesicle only were determined. A ratio of silver grains over the sex vesicle to silver grains over the entire nucleus minus the background counts of silver grains were compared in XO*Sxr*, XY*Sxr*, and XY^{+} mice using a Student's t test and a Kruskal-Wallis One-way ANOVA.

In vitro Fertilization

Techniques of *in vitro* fertilization were as described by Kot and Handel (1987). Female mice were given an injection of 5IU pregnant mare's serum gonadotropin (PMSG, Sigma) followed 48-50 hours later by an injection of 5IU human chorionic gonadotropin (hCG, Sigma). These superovulated females were killed by cervical dislocation approximately 12 hours post-hCG injection. The oviducts were removed, the ampullae ruptured, and unfertilized eggs collected in a drop of Whitten's Medium (WM; Hoppe and Pitts, 1973) with 1mg/ml hyaluronidase

(Sigma). After dispersal of cumulus cells, eggs were washed three times in 1ml aliquots of WM. Ten to twenty eggs were then placed in dishes which contained a 0.5ml aliquot of WM overlaid with paraffin oil.

Male mice were killed by cervical dislocation, the reproductive tracts removed, and the cauda epididymis and vas deferens dissected from the rest of the tissue. Sperm were gently squeezed from the cauda epididymis and vas deferens into 1ml WM. Sperm from two experimental males were pooled, whereas sperm from only one control animal was collected and a sperm count of each aliquot determined using a hemocytometer. The remaining suspensions were overlaid with paraffin oil equilibrated with medium and incubated at 37°C in an atmosphere of 5% CO_2 in air until the eggs were collected.

Sufficient sperm suspension was added to the fertilization dishes to achieve a final concentration of $1x10^6$ sperm per ml. Dishes were incubated at 37°C in 5% CO_2 in air. Addition of sperm to the fertilization dishes constituted the time of insemination.

At 3 or 4 hours after insemination, eggs were removed from the dishes and washed through two changes of fresh WM by gently pipetting through a pipette with a bore of approximately 250μm. This procedure removed attached, but not bound, sperm. After washing, eggs were pipetted into a drop of 2.5% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed in 10% neutral formalin, washed in distilled water, dehydrated, and stained with 0.25% lacmoid in 45% acetic acid (Toyoda and Chang, 1974). The eggs were then examined by phase-contrast

microscopy. The number and morphological type of sperm bound to the zona pellucida of the egg was determined. Scoring criteria were those of Kot and Handel (1987).

III. RESULTS

Sertoli Cells and Germ Cells of XX*Sxr* Mice

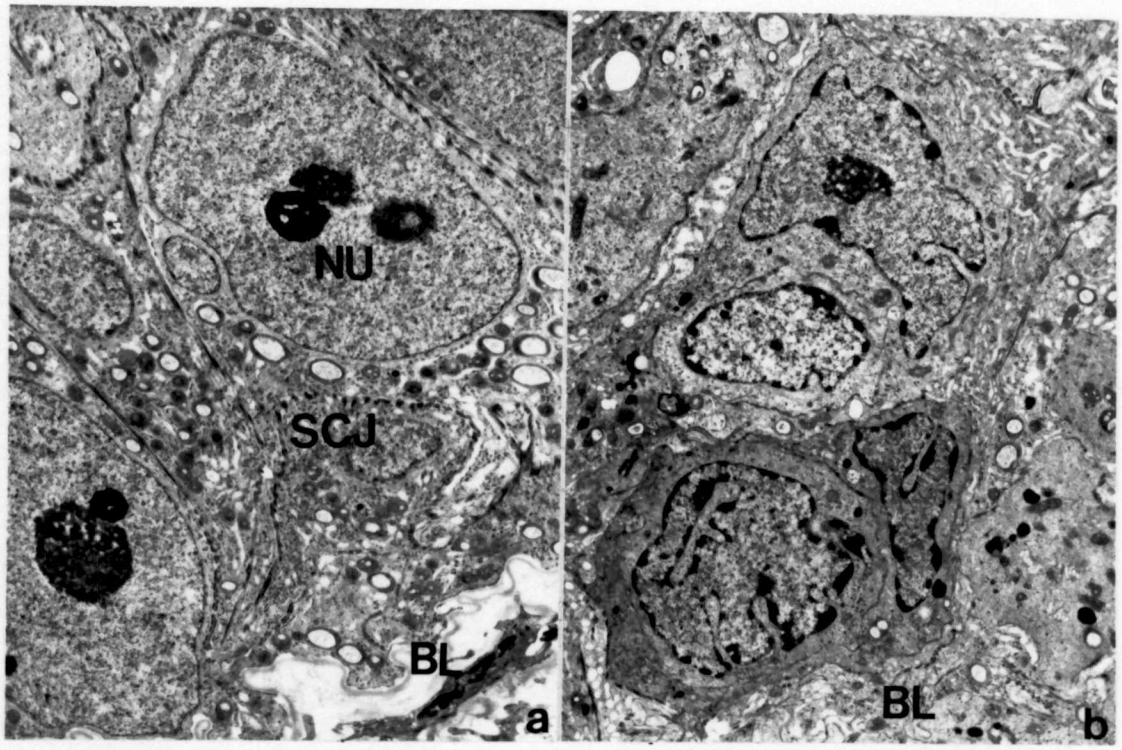
Although the focus of this investigation was XO*Sxr* mice, XX*Sxr* mice were examined for comparison. Three XX*Sxr* mice were analyzed by electron microscopy and four by light microscopy.

The testes of XX*Sxr* mice were characterized by the virtual absence of germ cells, although on rare occasions spermatogonia were present. Thus, in most cases, the seminiferous tubules contained only Sertoli cells. Many of these cells exhibited features characteristic of normal Sertoli cells, namely, tripartite nucleoli, nuclear infoldings, and characteristic Sertoli cell junctions (Fig. 3a). Some Sertoli cells of XX*Sxr* mice had ultrastructural abnormalities, such as large and oval, but not infolded, nuclear profiles; mitochondria with large, circular cristae; and decreased numbers of lipid droplets. A second type of somatic cell present within the seminiferous tubules of XX*Sxr* mice had small nuclei, no tripartite nucleoli, peripheral clumps of heterochromatin along the nuclear membrane, numerous vacuoles, and no typical Sertoli cell junctions (Fig. 3b). These anomalous cells were usually found in clusters and may be either atypical Sertoli cells or macrophages (Fig. 3b). Spermatogonia were seen rarely in ultrastructural analyses and never in light microscope analyses. They were located on the basal lamina adjacent to Sertoli cells, had oval

Figure 3. Somatic Cells of the Seminiferous Tubules of XX<u>Sxr</u> Mice.

a. Sertoli cells. Note the presence of a tripartite nucleolus (Nu), irregularly shaped nuclear envelope, typical Sertoli cell junctions (SCJ), and their location adjacent to the basal lamina (BL). X5250.

b. Type 2 somatic cells. Note the presence of small nuclei, peripheral clumps of heterochromatin within the nuclei, and the absence of tripartite nucleoli and typical Sertoli cell junctions. These cells are often located adjacent to the basal lamina (BL). X4375.


NU
SCJ
BL
a
BL
b

to round nuclei, finely granular nucleoplasm with small clumps of heterochromatin, and mitochondria typical of spermatogenic cells (Fig. 4).

Quantitative analyses of seminiferous tubules from XXSxr mice revealed that approximately 70% of the tubules contained both Sertoli cells and Type 2 somatic cells while 30% of the tubules contained only Sertoli cells (Table 1). The mean number of Sertoli cells per tubule was 20.1, whereas the mean number of the Type 2 somatic cell was 6.97 cells per tubule. Finally the quantitative studies showed that Sertoli cells represented approximately 74% of the cells in the seminiferous tubules of XXSxr mice (Table 1). Spermatogonia were not observed in the seminiferous tubule cross sections examined by light microscopy.

In summary, seminiferous tubules of XXSxr mice contained three different types of cells: Sertoli cells, anomalous Type 2 somatic cells of unknown origin (perhaps macrophages), and, rarely, spermatogonia. The major cell type of XXSxr tubules were Sertoli cells.

Kinetics of Spermatogenesis in Sxr Mice

Five animals of each genotype (XOSxr, XYSxr, and XY^{+}) were used to determine the kinetics of spermatogenesis in mice bearing the Sxr region. The number and type of spermatogonia, spermatocytes, and spermatids were determined from PAS/hematoxylin-stained testis sections and normalized to numbers of Sertoli cells (Table 2; Fig. 5).

Figure 4. Spermatogonia in the Seminiferous Tubules of XX<u>Sxr</u> Mice. Note the round nuclei with small clumps of heterochromatin and mitochondria typical of spermatogenic cells. These cells are located adjacent to the basal lamina (BL) and Sertoli cells. X3930.

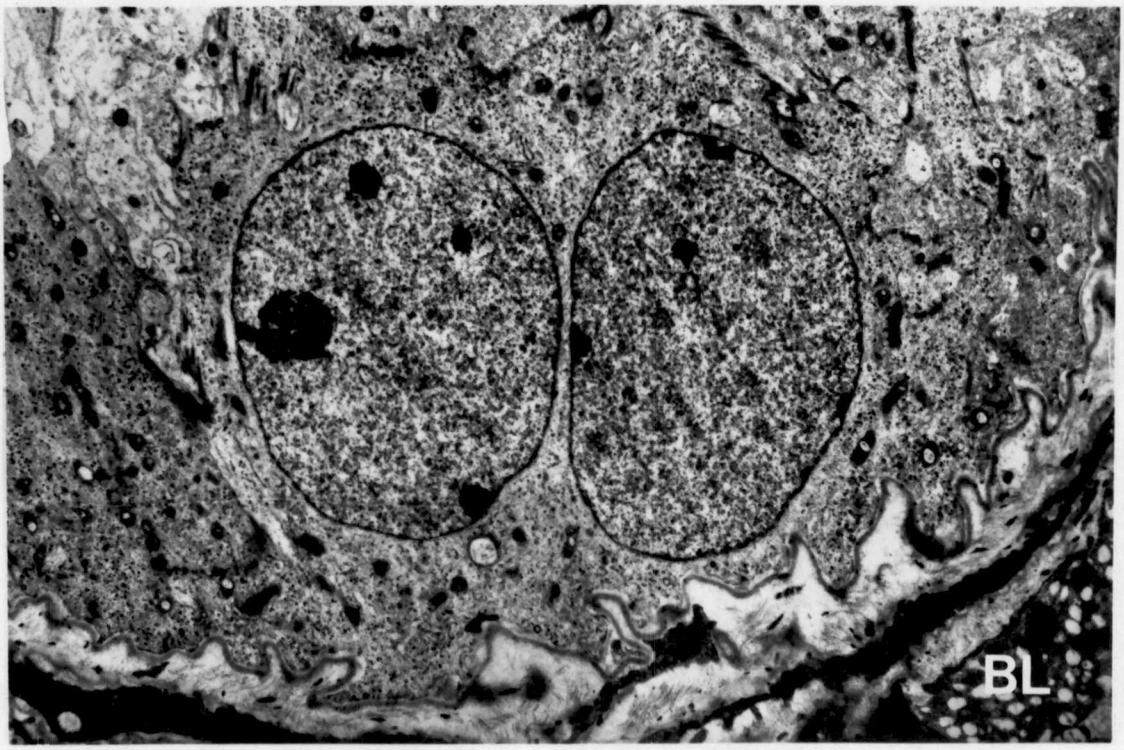

BL

Table 1. Cells within the Seminiferous Tubules of XXSxr Mice.

Mouse Number	Tubules with Sertoli and Type 2 Somatic Cells	Tubules with Sertoli Cells Only	Mean Number of Sertoli Cells Per Tubule	Mean Number of Type 2 Somatic Cells Per Tubule	Percentage of Type 2 Somatic Cells Within A Tubule
198	16/25 64%	9/25 36%	18.76±4.1	4.88±2.4	20.6%
204	20/25 80%	5/25 20%	15.44±5.3	6.40±4.1	29.3%
213	15/25 60%	10/25 40%	23.56±7.8	9.3±5.4	28.3%
218	19/25 76%	6/25 24%	22.6±6.7	7.3±6.5	24.4%
Mean	70%	30%	20.09	6.97	25.76%

Table 2. Relative Numbers of Differentiating Spermatogenic Cells in XO*Sxr*, XY*Sxr*, and XY Mice.

Stage of Differentiation	Mean Number per Sertoli Cell[1].			Comparison[2].	
	XY	XY*Sxr*	XO*Sxr*	XO*Sxr* vs XY	XO*Sxr* vs XY*Sxr*
Type A Spermatogonia	1.07	0.81	0.91	NS[3].	NS
Intermediate Spermatogonia	2.11	1.66	1.64	NS	NS
Type B Spermatogonia	3.04	2.05	2.33	NS	NS
Preleptotene Spermatocytes	5.09	4.06	3.27	$p < 0.01$	NS
Leptotene Spermatocytes	5.80	4.35	3.95	NS	NS
Zygotene Spermatocytes	6.06	4.67	3.50	$p < 0.01$	NS
Pachytene Spermatocytes	6.29	3.90	4.31	$p < 0.001$	NS
Diplotene Spermatocytes	7.34	2.15	3.23	$p < 0.01$	NS
MI Spermatocytes	1.34	1.26	1.57	NS	NS
MII Spermatocytes	0.52	0.42	0.87	NS	NS
Golgi Spermatids	15.51	4.27	2.62	$p < 0.0001$	NS[4].
Cap Spermatids	14.85	5.00	2.13	$p < 0.0001$	NS[4].
Acrosome Spermatids	16.12	4.15	2.04	$p < 0.0001$	NS[4].
Elongating Spermatids	15.75	4.66	2.15	$p < 0.0001$	$p < 0.01$
Mature Spermatids	11.64	3.21	1.21	$p < 0.0001$	NS[4].

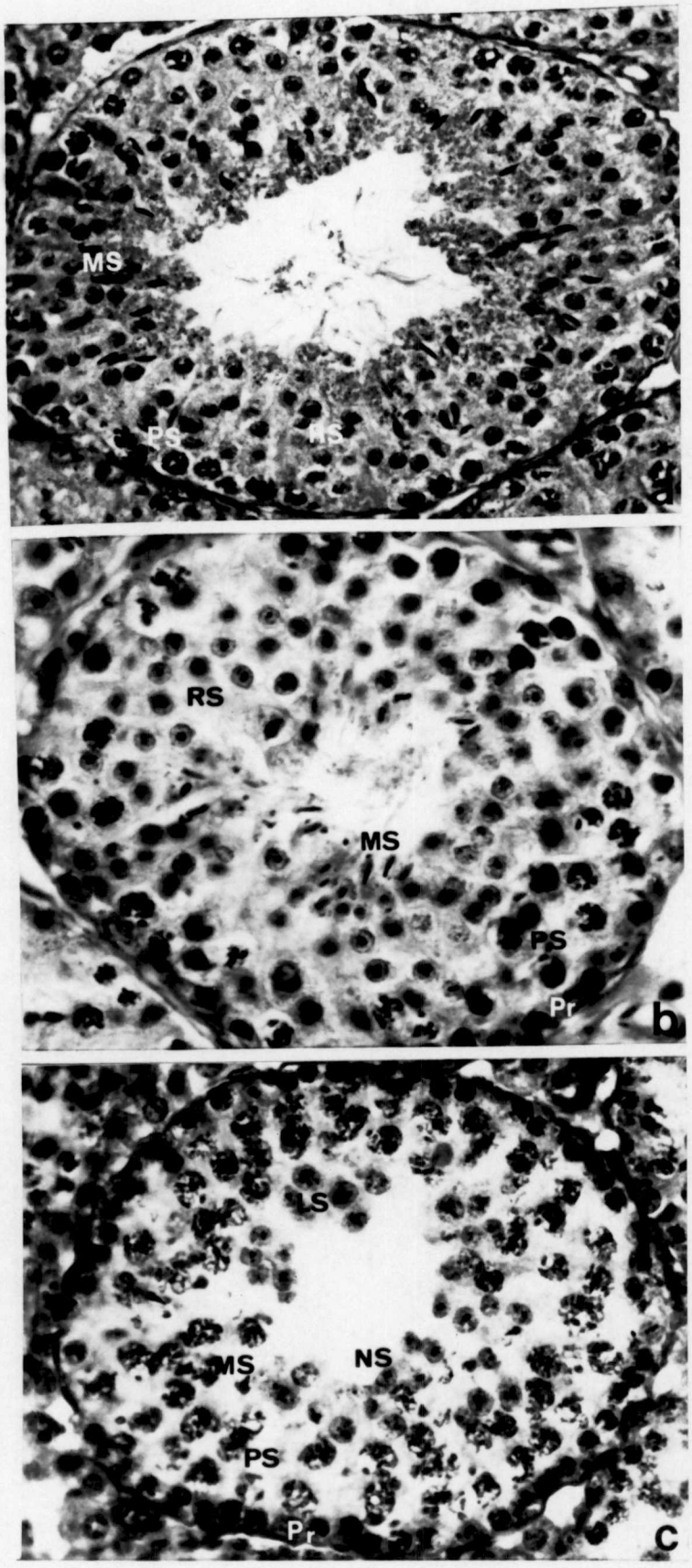
1. Cell numbers were determined for each stage of the seminiferous epithelium and averaged across all stages in which a cell type is represented.
2. Differences tested by ANOVA.
3. No significant difference.
4. For two age-matched mice from each group, difference is significant, $p<.01$; ANOVA.

Figure 5. Sections of Stage 7 Seminiferous Tubules Stained with PAS and Hematoxylin.

a. Section of a seminiferous tubule from an XY^{+} mouse. Note pachytene spermatocytes (PS), round spermatids (RS), and mature spermatids (MS). X450.

b. Section of a seminiferous tubule from an XY<u>Sxr</u> mouse. Note the preleptotene spermatocytes (Pr), pachytene spermatocytes (PS), round spermatids (RS), and mature spermatids (MS). X450.

c. Section of a seminiferous tubule from an XO<u>Sxr</u> mouse with preleptotene spermatocytes (Pr), pachytene spermatocytes (PS), normal-sized round spermatids (NS), large round spermatids (LS), and an occasional mature spermatid (MS). X430.


MS
PS
RS
MS
PS
Pr
b
LS
MS
NS
PS
Pr
c

There were no significant differences in the overall number of Type A, Intermediate, or Type B spermatogonia in XO<u>Sxr</u>, XY<u>Sxr</u>, and XY^+ mice as determined by an analysis of variance (ANOVA; Table 2). However, the number of spermatocytes in prophase I of meiosis I from both XO<u>Sxr</u> and XY<u>Sxr</u> mice showed a decrease when compared to the numbers from XY^+ mice. A 40% decrease in the number of prophase I spermatocytes was observed in XO<u>Sxr</u> mice, whereas a 35% decrease was observed in XY<u>Sxr</u> mice. These decreases reflected a significant difference between prophase I spermatocytes from XO<u>Sxr</u>, as well as XY<u>Sxr</u>, and XY^+ mice (ANOVA; $p<.01$; Table 2). One exception to this was noted; the overall numbers of leptotene spermatocytes were not significantly different among the three genotypes (ANOVA; $p<0.07$; Table 2). However, since preleptotene spermatocytes and zygotene spermatocytes (which are meiotic stages immediately preceding and following the leptotene stage) were both significantly reduced in XO<u>Sxr</u> and XY<u>Sxr</u> mice when compared to XY^+ mice, it is reasonable to conclude that the lack of statistical difference in the leptotene stage was due to a Type II statistical error (failure to demonstrate significance, probably because of variation or generally low numbers of this cell type). Further analysis of the kinetics of spermatocytes revealed that metaphase I (MI) and metaphase II (MII) spermatocytes from XO<u>Sxr</u> and XY<u>Sxr</u> were not significantly different from MI and MII spermatocytes in XY^+ mice (Table 2). Since these cell types were reduced in number and the cells immediately preceding (diplotene spermatocytes) and following (spermatids) them were significantly reduced, it is possible

that a Type II statistical error was made (especially given the small sample size and the small number of these cells present in normal mice). Spermatids from XO<u>Sxr</u> and XY<u>Sxr</u> were greatly reduced in number when compared with those observed in normal XY^{+} mice. XO<u>Sxr</u> reflected a 86% decrease and XY<u>Sxr</u> a 71% decrease from numbers present in XY mice (Table 2). These relative decreases were constant throughout spermiogenesis and were statistically significant (ANOVA; $p<0.01$; Table 2).

The dramatic decreases in numbers of meiotic and post-meiotic cells from XY<u>Sxr</u> mice was unexpected since these mice are fertile. One possible problem in this analysis is that the XO<u>Sxr</u> and XY<u>Sxr</u> mice used in the analysis were not age-matched. This problem was unavoidable since the availability of both genotypes of mice was extremely limited and the XY<u>Sxr</u> mice were used for breeding prior to analysis. Nonetheless, analysis of cell numbers relative to age revealed that the age of the mice used in the study could be a major factor contributing to the reduction in spermatid number. Therefore, an analysis of variance was used to compare a smaller sample of XO<u>Sxr</u> and XY<u>Sxr</u> mice that were more closely matched for age. The numbers of spermatogonia and spermatocytes were not significantly different in age-matched XO<u>Sxr</u> and XY<u>Sxr</u> mice (Table 2). However, post-meiotic cells were significantly lower in XO<u>Sxr</u> mice than in XY<u>Sxr</u> mice of approximately the same age (ANOVA; $p<.01$; Table 2). Older XY<u>Sxr</u> mice seemed to have a greater reduction in the number of post-meiotic germ cells (but not of meiotic cells) than younger XY<u>Sxr</u> mice. It was

concluded that age is a contributing factor to germ-cell loss in XY<u>Sxr</u> mice.

One hundred-twenty five seminiferous tubules per genotype (25 tubules per mouse for five mice per genotype) were scored for the presence of degenerating cells (Table 3). In XO<u>Sxr</u> mice 24% of the seminiferous tubules scored contained degenerating cells (Table 3). XY<u>Sxr</u> and XY^+ mice exhibited lower percentages of seminiferous tubules containing degenerating cells (15.2% and 4%, respectively; Table 3). Degenerating cells were most commonly found in stage 12 of all three genotypes, and the degenerating cells appeared to be in metaphase.

In summary, spermatogonia were present in normal numbers in XO<u>Sxr</u> and XY<u>Sxr</u> mice. There were significant reductions in numbers of most primary spermatocytes in XO<u>Sxr</u> and XY<u>Sxr</u> mice, as compared to XY^+ mice. Furthermore, a significant reduction in numbers of all spermiogenic cells was observed in XO<u>Sxr</u> and XY<u>Sxr</u> mice. When XO<u>Sxr</u> and XY<u>Sxr</u> mice were age-matched, XO<u>Sxr</u> mice had significantly lower numbers of spermatids. In addition, it was found that age contributed to spermatid loss in XY<u>Sxr</u> males. Tubules containing degenerating cells were present in all genotypes examined. These tubules were most commonly found in XO<u>Sxr</u> mice, were usually associated with stage 12, and contained degenerating metaphase cells.

Table 3. Degenerating Cells in Seminiferous Tubules of XO*Sxr*, XY*Sxr*, and XY Mice.

	Number of Tubules with No Degenerating Cells	Number of Tubules with Degenerating Cells	Percentage of Tubules with Degenerating Cells	Mean Number of Degenerating Cells Per Tubule
XY	120	5	4%	5.0±5.0
XY*Sxr*	106	19	15.2%	2.6±1.1
XO*Sxr*	95	30	24%	3.3±1.8

Structural Features of Sertoli and Spermatogenic Cells of Sxr Mice

Testes from five XYSxr, three XOSxr, and five normal XY mice were examined by electron microscopy.

Sertoli Cells. Typical Sertoli cells were observed in the testes of XOSxr mice as compared to XYSxr and XY^{+} mice. These cells, located on the basal lamina of seminiferous tubules, had nuclei with characteristic infoldings, granular nucleoplasm, and a prominent tripartite nucleolus consisting of a thread-like nucleolema portion and two compact portions (Fig. 6a). Mitochondria with tubular cristae, smooth endoplasmic reticulum (SER), and densely staining lipid droplets, as well as residual bodies, were observed in the cytoplasm. Elongating spermatids were often observed phagocytosed within the Sertoli cell cytoplasm from XOSxr mice. Specialized Sertoli cell junctions, a type of tight junction with thickened areas where two membranes are juxtaposed and associated with bundles of filaments and tubular elements (Fig. 6b), were present between adjacent cells. The anomalous Type 2 somatic cell found in the tubules of XXSxr mice was not found in XOSxr or XYSxr mice.

Spermatogonia. Spermatogonia from XOSxr, XYSxr, and normal XY males had similar structure. They were located on the basal lamina adjacent to the Sertoli cells (Fig. 7). Nuclei of Type A spermatogonia were oval-shaped, finely granular, and contained nucleoli which were coarse, thread-like networks (Fig. 7a). Mitochondria were oval-shaped structures with closely spaced cristae. Intercellular, cytoplasmic bridges were present between adjacent

Figure 6. Sertoli Cells of XO<u>Sxr</u> Mice.

a. Typical Sertoli cell with prominent tripartite nucleolus (Nu) within the nucleus. X5145.

b. Specialized Sertoli cell junctions. The junction (J) has associated and underlying filaments and tubular elements. Also present are mitochondria (M) and densely staining lipid droplets (LD). X56,000.

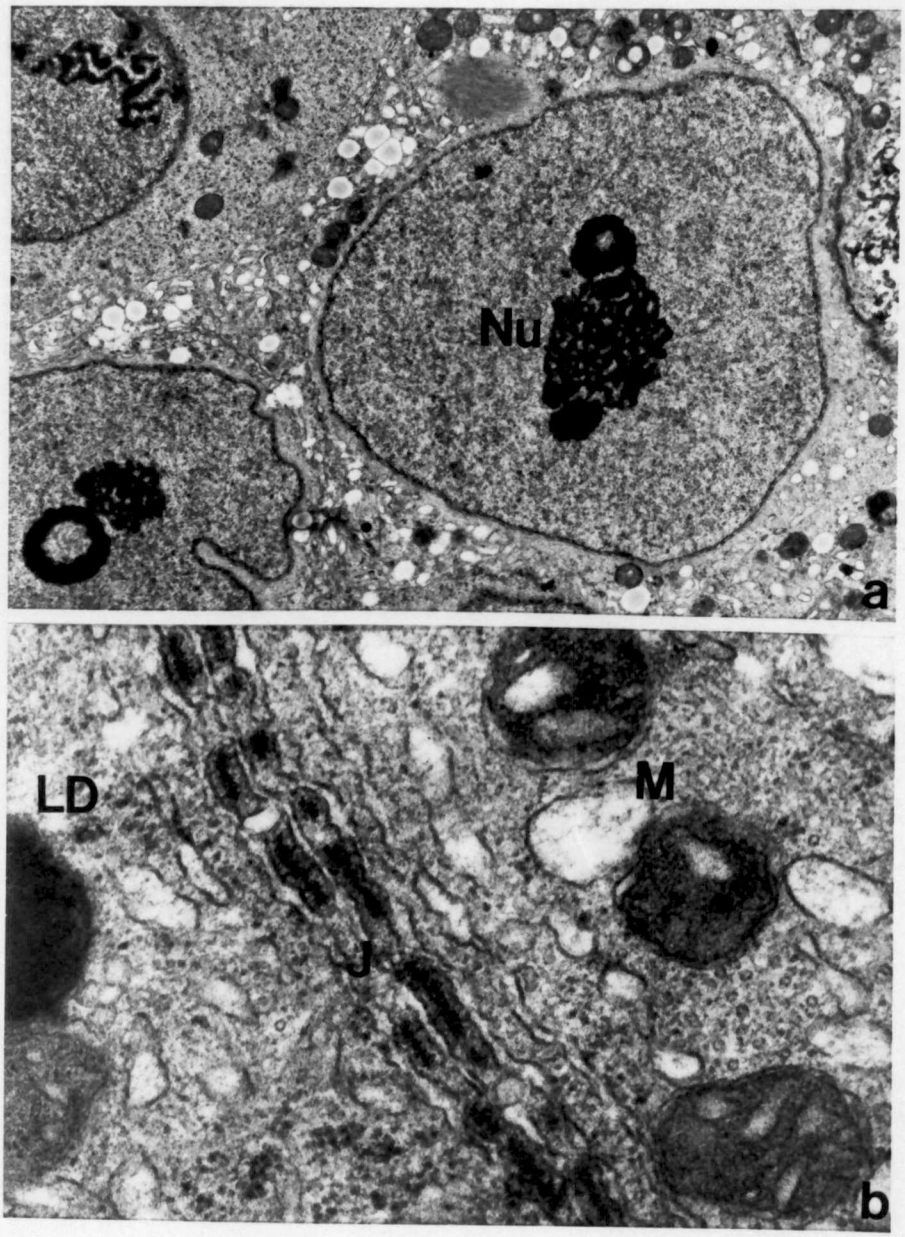
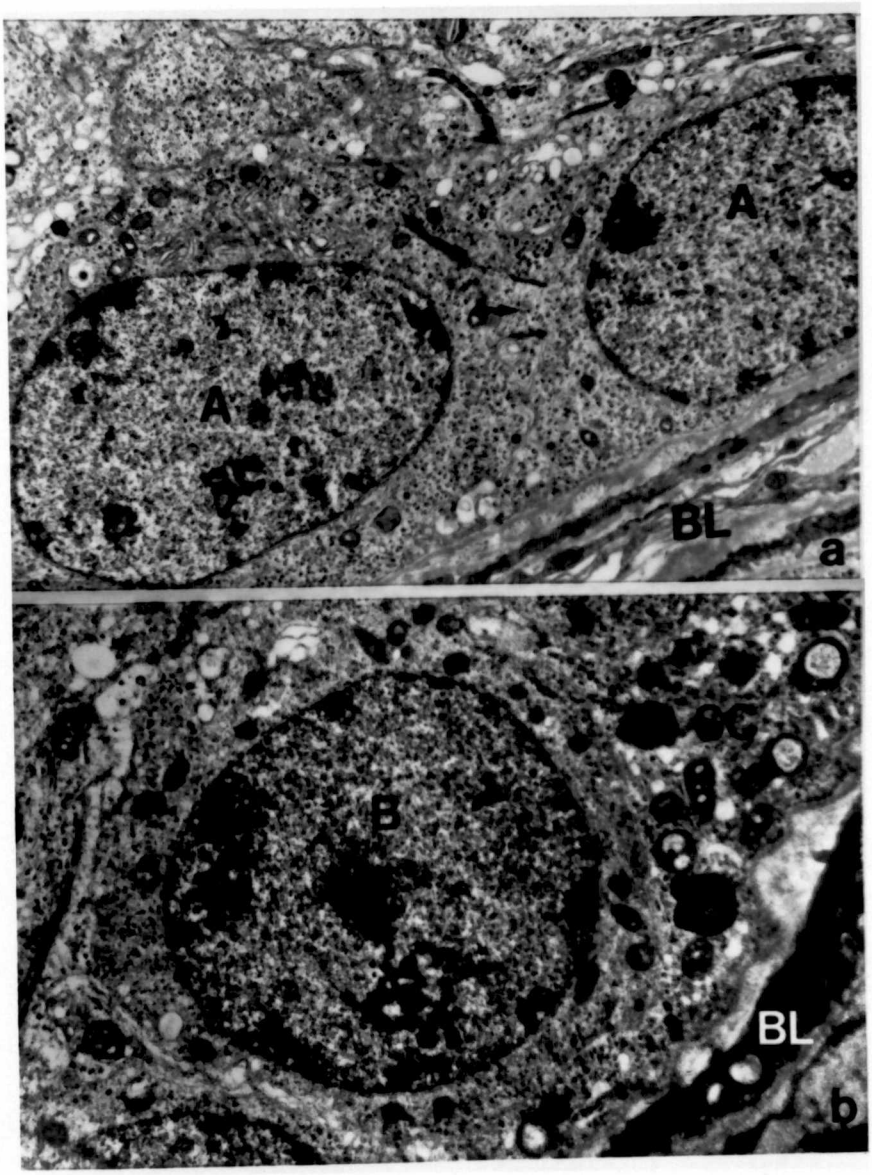

Nu
a
LD
M
J
b

Figure 7. Spermatogonia of XO<u>Sxr</u> Mice.

a. Two Type A spermatogonia (A) connected by a cytoplasmic bridge (arrow). Note heterochromatic clumps within the nuclei and the location of the cells with respect to the basal lamina (BL). X7160.

b. A Type B spermatogonium (B). The cell is adjacent to Sertoli cell cytoplasm (SC) and the basal lamina (BL). X6750.


A
A
BL
a
B
BL
b

spermatogonia (Fig. 7a) and were characterized by a dense specializations of the membrane. Nuclei of Type B spermatogonia were round, with a finely granular nucleoplasm, and contained several diffuse, thread-like nucleoli which were often centrally located (Fig. 7b). Mitochondria, which were evenly distributed throughout the cytoplasm, were round, and had few cristae.

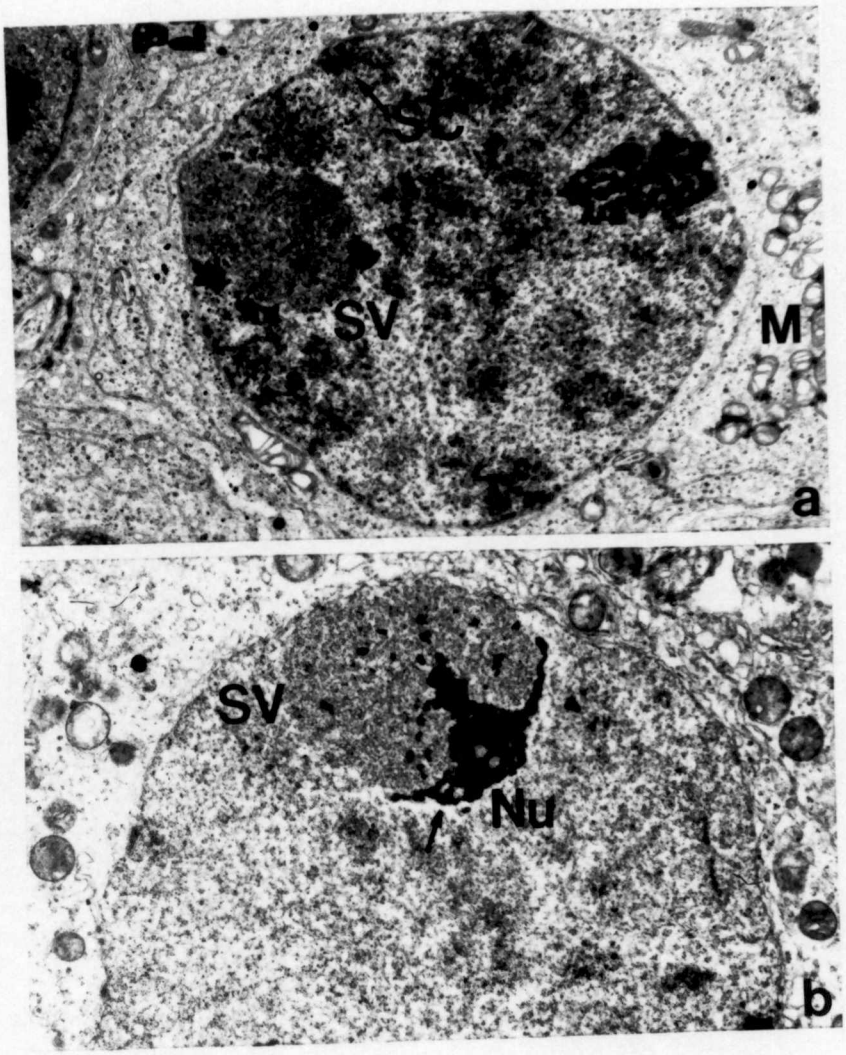
Spermatocytes. Leptotene and zygotene spermatocytes of XOSxr mice appeared morphologically normal when compared to those of XYSxr and XY+ mice. The leptotene spermatocyte nuclei were round with finely granular chromatin and a single nucleolus. Adjacent leptotene spermatocytes were connected by intercellular bridges. Zygotene spermatocyte nuclei were characterized by a round shape and random patches of condensed chromatin. Intercellular bridges were observed between adjacent zygotene spermatocytes.

The nuclei of pachytene spermatocytes of XY and XYSxr males were characterized by a granular, but unevenly condensed appearance, a round to oval shape, and a sex vesicle (Fig. 8). The sex vesicle was round or oval with an average perimeter of 3.35±.3μm in XY mice and 3.45±.4μm in XYSxr mice. It stained more darkly than the rest of the nucleus, was evenly condensed, and was granular. The sex vesicle was apposed to the nuclear envelope along its outer edge and, in late spermatocytes, had a crescent-shaped nucleolus along its inner, nucleoplasmic surface (Fig. 8b). The nucleolus was characterized by a trabecular, thread-like structure with two or more arms extending from a round, central body. Occassionally, it was observed that within the

Figure 8. Pachytene Spermatocytes.

a. Pachytene spermatocyte from an XY<u>Sxr</u> mouse. Note the presence of a sex vesicle (SV) within the nucleus, as well as synaptonemal complexes (SC) attached to the nuclear envelope. Also note the presence of typical spermatocyte mitochondria (M) within the cytoplasm. X6160.

b. Pachytene spermatocyte nucleus from an XY^{+} mouse with a prominent sex vesicle (SV) and crescent-shaped nucleolus (Nu) with a central element and a trabecular portion (arrow). X9300.


SV
M
a
SV
Nu
b

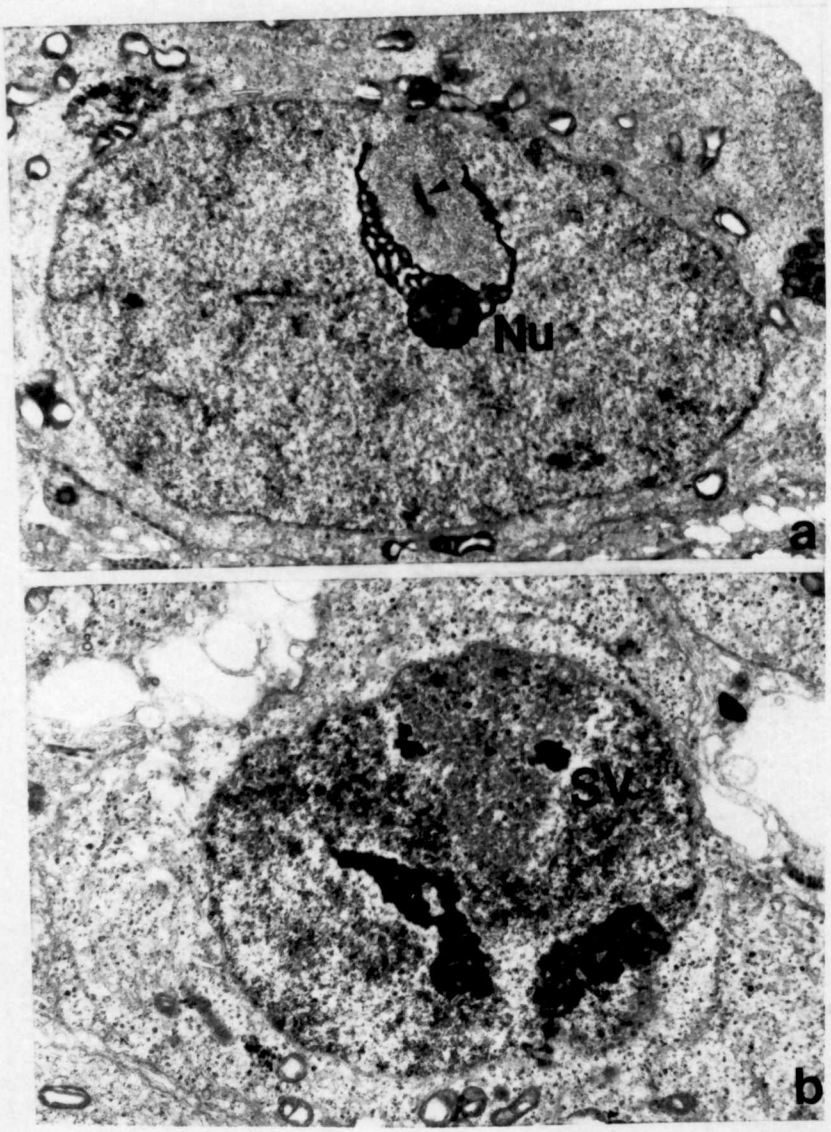
sex vesicle the X and Y chromosomes formed a synaptonemal complex along part of their lengths. This structure was trilaminar, composed of two lateral elements associated with chromatin and a central bar. During late pachynema basal knobs of both the autosomes and sex chromosomes were visible along the nuclear envelope. The dumb-bell or oval-shaped mitochondria of these cells had large spaces between the cristae. Abundant SER was present at the periphery of the cell. Intercellular bridges were found between primary spermatocytes.

XO<u>Sxr</u> mice had pachytene spermatocytes in which the nucleus was round to oval, granular, unevenly condensed, and characterized by the presence of a sex vesicle (Fig. 9). The sex vesicle was usually normal in shape, had an average perimeter of 3.38$\pm$.2 μm (not different than that of control mice; Student's t test), showed the usual relationship to the nuclear envelope and nucleolus, and was evenly condensed and granular (Fig. 9a). Only occasionally were profiles of synaptonemal complex observed within the sex vesicle (Fig. 9a). Occasional abnormalities were observed: the sex vesicle of some pachytene spermatocytes was triangular rather than round or oval, and the nuclei of some cells were irregularly shaped and indented (Fig. 9b). Autosomal basal knobs were seen in late pachytene spermatocytes. Mitochondria, SER, and intercellular bridges were present and appeared normal.

Figure 9. Pachytene Spermatocytes of XO<u>Sxr</u> Mice.

a. Morphologically normal pachytene spermatocytes with a synaptonemal complex (arrow head) within the sex vesicle and a crescent-shaped nucleolus (Nu) associated with the sex vesicle. X7500.

b. Morphologically abnormal pachytene spermatocyte. Note the abnormal shape of the sex vesicle (SV). X7200.


Nu
a
SV
b

Spermatids: Two distinct populations of spermatids were present in XOSxr and XYSxr mice; large spermatids and normal-sized spermatids (Fig. 5, page 32). These two populations were distinct in that they differed by three standard deviations, and were significantly different from each other at each stage of spermiogenesis (Student's t test; $p<.01$; Table 4). Large spermatids were not present in XY^+ controls (Tables 4 and 5). Approximately 23.5% of all stages of spermatids from XOSxr mice were significantly larger than the normal-sized spermatids (Tables 4 and 5), while approximately 2.2% of all XYSxr spermatids were of the large size-class. The percentage of large spermatids did not change significantly from Golgi-stage round spermatids to elongating spermatids (dependent t test), indicating that the large spermatids were not preferentially degraded or otherwise lost from the seminiferous epithelium.

Ultrastructural analyses of the XOSxr mice revealed both normal and abnormal round spermatids as compared to normal XY mice. Golgi-stage spermatids of XOSxr had round nuclei which contained granular chromatin and clumps of heterochromatin. The Golgi apparatus was located directly apical, and often to one side, of the forming acrosomic vesicle. The round acrosomic vesicle caused the nuclear envelope to indent and thicken (Fig. 10a). A densely staining acrosomal granule was often present within the vesicle (Fig. 10a). Round to oval mitochondria with large spaces between the cristae were found at the periphery of the cells (Fig. 10a). Cap and acrosomal stage spermatids had a normal-appearing spermatid tail attached at the

Table 4. Perimeters of Spermatid Nuclei from XOSxr, XYSxr, and XY Mice[1].

Mouse Type	Golgi Stage Round Spermatids		Cap Stage Round Spermatids		Acrosomal Stage Round Spermatids		Elongating Spermatids		Mature Spermatids	
	Normal Size	Large[2] Size	Normal Size	Large Size	Normal Size	Large Size	Normal Size	Large Size	Normal Size	Large Size
XY	18.70± 1.66	ND[3]	18.73± 1.66	ND	18.99± 1.68	ND	13.42± 3.07	ND	9.72± 2.22	ND
XYSxr	18.64± 1.69	35.33± 5.23	18.93± 1.61	35.46± 3.63	19.24± 3.25	35.95± 3.78	13.71± 3.10	32.84± 7.18	7.46± 1.41	19.88± 6.01
XOSxr	18.84± 1.66	37.53± 4.23	18.88± 1.74	36.52± 4.38	18.77± 1.70	35.36± 3.73	13.35± 6.33	30.74± 5.86	8.04± 1.44	18.73± 2.61

[1]All values expressed as μm.
[2]Large size class is defined as >3SD above the normal size XY mice and is significantly different from normal ($p<.01$, d.f. = 4, dependent t test).
[3]None detected.

Table 5. Percentage of Large Spermatids in XO*Sxr*, XY*Sxr*, and XY Mice.

Mouse Type	Round Spermatids			Elongating Spermatids	Mature Spermatids
	Golgi	Cap	Acrosomal		
XY	0	0	0	0	0
XY*Sxr*	3.94	1.63	3.35	1.81	0.26
XO*Sxr*	29.82	30.09	32.95	19.02	5.79

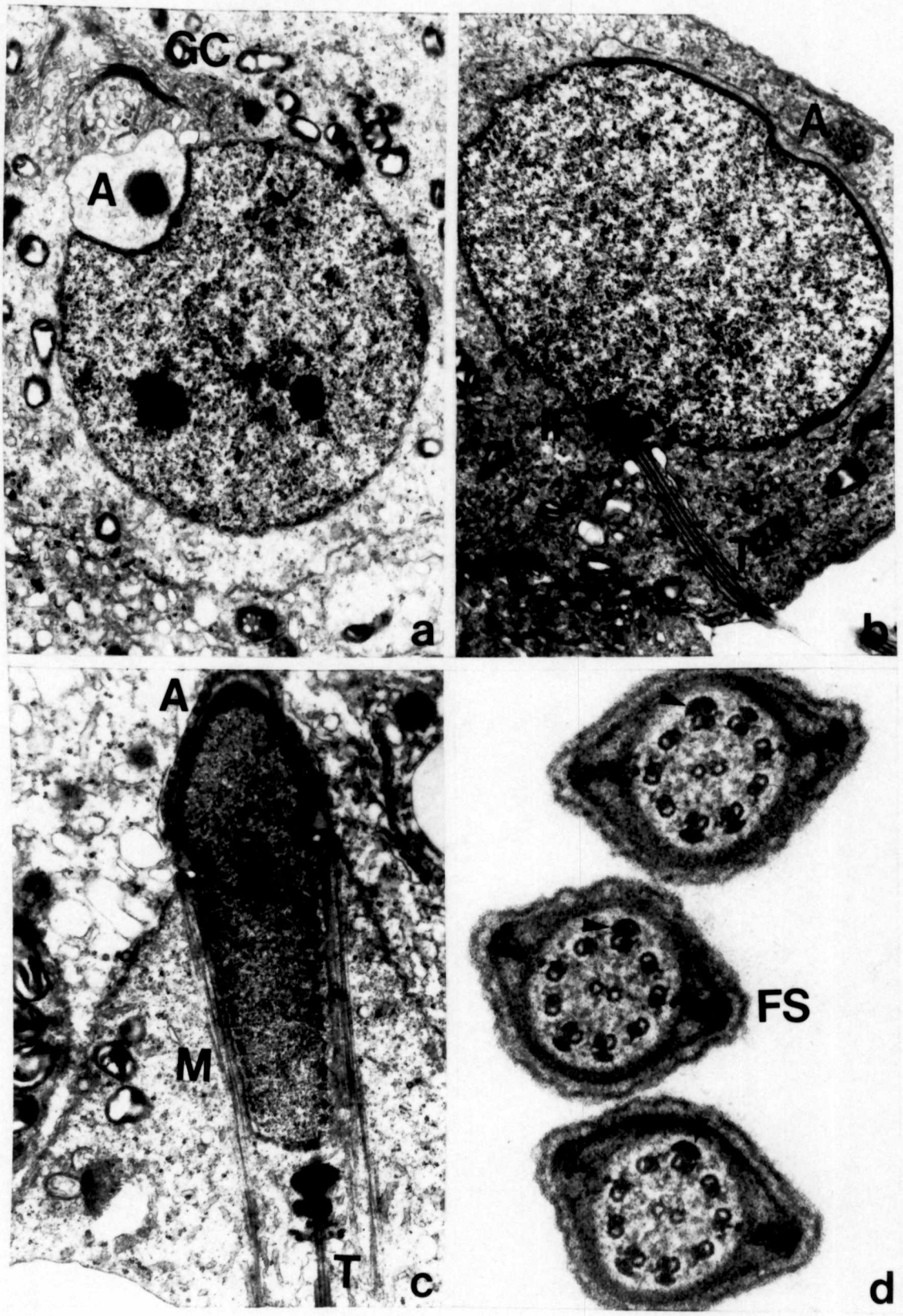
Figure 10. Morphologically Normal Spermatids of XO<u>Sxr</u> Mice.

a. Golgi-stage round spermatid with an acrosomic vesicle (A) and acrosome-associated Golgi Complex (GC). X7670.

b. Acrosomal-stage round spermatid with an acrosome (A), tail (T), and attachment at implantation fossa (IF). X8000.

c. Elongating spermatid with an acrosome (A), microtubular manchette (M), and tail (T). X9020.

d. Cross section of spermatid tails. Note the presence of axonemal structure, outer dense fibers (arrow head) and the fibrous sheath (FS). X90,000.


GC
A
a
A
T
b
A
M
T
c
FS
d

implantation fossa of the nucleus (Fig. 10b). The tail and acrosome assumed opposite positions in these spermatids. The thickened region of the nuclear membrane underlying the acrosome had spread caudally in concert with the acrosome (Fig. 10b). A chromatoid body was present. Mitochondria were round or slightly oval with several cristae running the length of the organelle. During the elongation phase of spermiogenesis, nuclei began to elongate and condense (Fig. 10c). At this stage, mitochondria were elongated ovals with circular cristae and were located in the area of the developing middlepiece of the tail. A manchette, consisting of parallel arrays of microtubules extending from a postacrosomal region to the principal piece of the tail, formed (Fig. 10c). Spermatid tails were characterized by the normal lengthwise differentiation: a midpiece, a principal piece, and an end piece. The central "9 + 2" arrangement of microtubular components of the axoneme appeared normal in all the segments. In the midpiece, which is the segment of the tail adjacent to the nucleus, the axoneme was surrounded by nine outer dense fibers, a mitochondrial sheath, and the cell membrane, as was expected. The principal piece, the longest segment, contained an axoneme, nine outer dense fibers, a fibrous sheath, and a cell membrane. The end piece, (Fig. 10d) was characterized by tapering and ending of the outer dense fibers, fibrous sheath, and an axoneme. In late, maturing spermatids nuclear condensation continued with small transparent areas within areas of more condensed chromatin. The head eventually assumed a fusiform or tapered shape with the area of tail attachment flattened.

Abnormal spermatids were observed frequently in the testes of XOSxr mice. The abnormal Golgi-stage spermatids had multiple proacrosomal granules and a nuclear envelope that was not indented or thickened (Fig. 11a). Cap and acrosomal-stage spermatids were observed that had double nuclei but a common acrosome (Fig. 11b). Although normal elongating and mature spermatids were present, a high frequency of spermatid-head abnormalities were observed in elongating and maturing spermatids (Figs. 11c and 11d). Some elongating and mature spermatids with condensed chromatin had double or bifurcated heads within a common cytoplasm and shared a common acrosome and manchette (Fig. 11c). Frequent aberrations of spermatid head shape were observed in XOSxr mice (Fig. 11d), including indentations, convoluted nuclear membranes and incomplete elongation of the sperm head. Many such abnormal spermatids appeared to be phagocytosed within the Sertoli cell cytoplasm. Even these aberrant spermatids had all sperm-specific structures: acrosomes, condensed nuclei, manchettes, and sperm tails (Figs. 11c and 11d). As previously noted, some seminiferous tubule cross-sections contained degenerating cells. The cells, found in clusters, were extremely large, had dense clumps of chromatin, and often appeared to be in metaphase. These cells were not seen in normal XY mice.

Normal and abnormal spermatids of all steps were observed in XYSxr mice. Like spermatids of XOSxr mice, spermatids of XYSxr mice contained normal acrosomes, condensed sperm heads, manchettes, and sperm tails. Degenerating cells and double-headed sperm of various

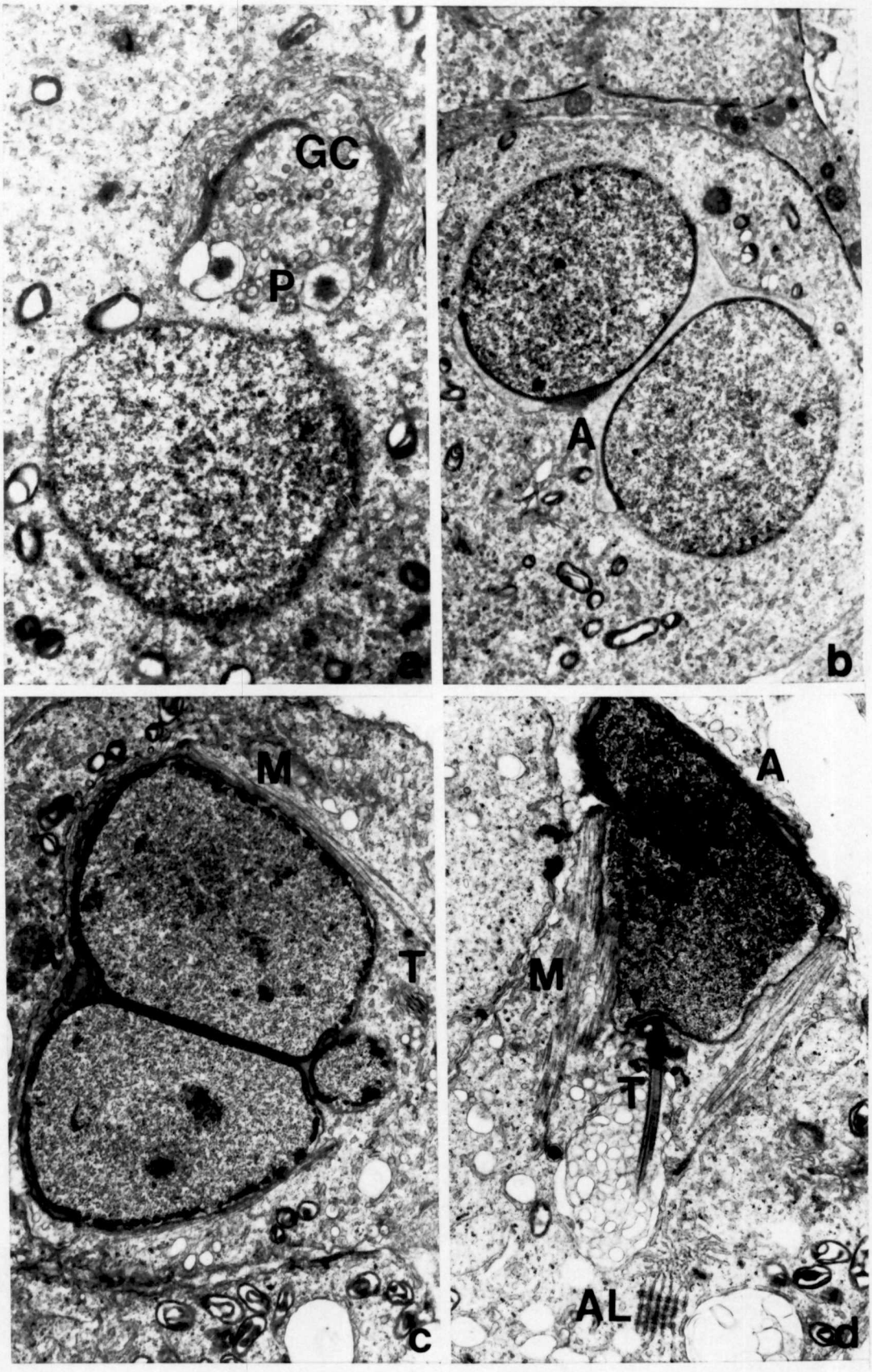
Figure 11. Morphologically Abnormal Spermatids of XOSxr Mice.

a. Golgi-stage round spermatid with multiple proacrosomal vesicles (P) and acrosome-associated Golgi complex (GC). X11700.

b. Acrosomal-stage round spermatid with two nuclei, each associated with a common acrosome (A). X5670.

c. Elongating spermatid. Note that the cell has two (or possibly three) nuclei and a common acrosome (A). However, the manchette (M), tail (T), and nuclear condensation appear normal. X8140.

d. Elongating spermatid. Note the aberrant morphology of the sperm head. Present are an acrosome (A), manchette (M), annulate lamellae (AL), condensed nucleus, implantation fossa, and tail (T). X9000.


GC
P
A
b
M
T
c
A
M
T
AL
d

stages similar to those in XO<u>Sxr</u> mice were observed in some tubule cross sections.

<u>Spermatozoa</u>: XO<u>Sxr</u> mice were characterized by an extremely high percentage of abnormal sperm (55% to 100%; Tables 6 and 7). XY<u>Sxr</u> and normal XY mice were characterized by moderately high levels of morphologically abnormal sperm (19% to 62% in XY<u>Sxr</u> and 12% to 33% in XY^+ mice; Tables 6 and 7). Large variability in the percentage of abnormal sperm was seen in each of the three genotypes (Table 6). In XY<u>Sxr</u> mice the percentage of sperm abnormalities increased with age. However, this was not true for XO<u>Sxr</u> or normal XY males (data not shown). The morphology of sperm from the cauda epididymis and vas deferens was classified according to the criteria of Kot and Handel (1987) and the distribution is presented in Table 7. Type 1 sperm (with elongated post-acrosomal areas), Type 7 sperm (with truncated tails but normal heads), and Type 8 sperm (with truncated tails and abnormal heads) were not observed in XO<u>Sxr</u>, XY<u>Sxr</u>, or normal XY mice. Type 2 sperm (lacking a rostrum), Type 3 sperm (with a broad post-acrosomal area), Type 4 sperm (with an oval shaped head), Type 5 sperm (with an amorphous head shape), Type 6 sperm (with a globular head shape), Type 9 (with highly coiled tails), and Type 10 (morphologically normal) were observed in XO<u>Sxr</u>, XY<u>Sxr</u>, and XY^+ mice. Four additional types of sperm categories were also present in these mice. First, sperm with two heads were observed (Type 11). Second, sperm with two or more tails (Type 12) were present in <u>Sxr</u>-bearing and normal mice. Third, sperm which had the head folded back onto the

Table 6. Percentages of Morphologically Abnormal Epididymal Spermatozoa in XO*Sxr*, XY*Sxr*, and XY Mice.

Mouse Phenotype	Mouse Number	Mean Percentage of Abnormal Epididymal Spermatozoa
XY	609	12.5
	107	25.8
	424	31.1
	248	32.8
XY*Sxr*	274	19.0
	208	27.2
	242	51.3
	153	62.0
XO*Sxr*	244	54.6
	1153	76.0
	Pooled Sample[1]	99.7
	1154	100.0

[1]Pooled sample from 3 XO*Sxr* mice.

Table 7. Percentage of Sperm in Various Morphological Categories in XO*Sxr*, XY*Sxr*, and XY Mice.

	Sperm Morphology Class[1]										
Mouse Type	2	3	4	5	6	9	10	11	12	13	14
XY	1.4	27.8	0	1.3	3.2	1.7	74.5	0.1	0.02	1.2	9.4
XY*Sxr*	2.5	4.5	0.1	1.6	2.4	2.1	76.9	0.3	0.2	4.5	4.9
XO*Sxr*	4.5	8.2	5.3	1.6	47.4	8.5	12.0	0.1	2.0	9.0	1.4

[1] According to Kot and Handel (1987) and as defined here.
Note: Types 1,7, and 8 were not observed in these genotypes.

midpiece or the tail (Type 13) were present. Fourth, sperm with heads and midpieces folded back onto the tail (Type 14) were present in XO<u>Sxr</u>, XY<u>Sxr</u> and normal XY mice. It is important to note that the abnormal sperm types observed were not unique to the <u>Sxr</u> mice but are found in a variety of male-sterile mouse mutants (Handel, 1987). The percentage of sperm in each category in XO<u>Sxr</u>, XY<u>Sxr</u> and XY^+ mice is presented in Table 7. Furthermore, each genotype (XO<u>Sxr</u>, XY<u>Sxr</u>, and XY^+) was characterized by a different distribution of morphologically abnormal sperm. XO<u>Sxr</u> sperm were predominantly Type 6, while XY<u>Sxr</u> and XY^+ mice had predominantly normal (Type 10) sperm.

Hemocytometer counts of sperm suspensions in WM were done to determine the number of motile sperm in XO<u>Sxr</u> and control mice. No sperm from XO<u>Sxr</u> mice were motile under these conditions. However, sperm from XY and XY<u>Sxr</u> mice do exhibit motility when incubated in WM medium (73.3% and 56.9%, respectively). In addition, hemacytometer counts revealed that XO<u>Sxr</u> mice have 1.04×10^6 epididymal sperm per ml; XY<u>Sxr</u> mice had 1.01×10^7 sperm per ml, and XY mice had 2.87×10^7 epididymal sperm per ml. Thus, XO<u>Sxr</u> mice were characterized by non-motile sperm and a sperm count 10% that of XY^+ mice, and XY<u>Sxr</u> mice had motile sperm and a sperm count approxiamtely 35% that of XY^+ mice.

In summary, Sertoli cells and spermatogonia were morphologically normal, whereas many of the primary spermatocytes of XO<u>Sxr</u> mice were not. However, the sex vesicle was present in both morphologically normal and abnormal pachytene spermatocytes. Furthermore, while morphologically normal spermatids were present within the testes of

XOSxr mice, abnormal spermatids were present as well. Abnormalities included such features as extremely large nuclei, multiple acrosomal granules, multiple nuclei, and convoluted nuclear membranes. Even aberrant spermatids had all sperm-specific strucutres. XOSxr mice were also characterized by high percentages of abnormal, nonmotile sperm and a sperm count which was only 10% that of XY^+ mice.

Biochemical Aspects of Spermatogenic Differentiation in Sxr Mice

Nuclear Transcription: Four mice of each genotype (XOSxr, XYSxr, and XY^+) were used to assess the transcriptional activity of the sex chromosomes by [^{3}H]-uridine incorporation, followed by autoradiography. The number of grains over the nuclei of pachytene spermatocytes, as well as the number of grains over the sex vesicles of these cells and equivalent background areas, were determined for two hundred cells per mouse. Due to the small size of the sex vesicle it was not possible to determine accurately an area equal to that of the sex vesicle for background counts. XOSxr and XYSxr mice had a higher percentage of sex vesicles with autoradiographic grains than did normal XY mice (36% and 46% respectively, compared to 31% for XY^+ mice; Table 8). In addition, the mean percentage of grains over the sex vesicles was higher in XOSxr and XYSxr mice (Table 8). However, these data were not corrected for the background grains. To correct for the presence of background grains, a ratio of the number of grains over the sex vesicle to the total number of grains over the nucleus

Table 8. Number of Autoradiographic Grains on the Sex Vesicles of Pachytene Spermatocytes.

Mouse Type	Mean Age in Months	Percentage of Labeled Sex Vesicles[2]	Range of Grains Over the Sex Vesicles[2]	Average Number of Grains per Sex Vesicle[2]
XY	6.25	31.38% (251/800)	0-6	0.55±.08 (437/800)
XY<u>Sxr</u>	17.5	46.12% (369/800)	0-8	0.96±.10 (764/800)
XO<u>Sxr</u>	7.75	36.00% (288/800)	0-10	0.67±.17 (533/800)

[1]Four mice from each genotype were used. 200 cells were scored per mouse.
[2]These data have not been corrected for background grain counts.

minus the background grain counts was used for each cell. To ensure that the ratio was a valid way to assess the incorporation of [^{3}H]-uridine into the sex vesicle, the number of grains over the entire nucleus minus the background was compared in XO<u>Sxr</u>, XY<u>Sxr</u>, and XY$^+$ mice utilizing the Student's t test that showed no significant difference between the total number of grains over the nucleus of pachytene spermatocytes in XO<u>Sxr</u> and XY$^+$ mice (after being corrected for background). There was a significantly higher number of grains over the nuclei of pachytene spermatocytes in XY<u>Sxr</u> mice than in normal XY mice ($p<.0001$; Student's t test). Although this was a significant difference, it did not adversely affect the validity of the ratio. By increasing the demoninator of the ratio (the total grain counts over the nucleus minus the background) the overall ratio would be lower. However, the ratio of the grains over the sex vesicle to grains over the entire nucleus was still significantly higher in XY<u>Sxr</u> mice than XY$^+$ mice ($p<.0007$; Kruskal-Wallis 1-Way ANOVA). In addition, the number of grains over the sex vesicle was found to be significantly higher in XO<u>Sxr</u> mice than in XY$^+$ mice ($p<.0007$; Kruskal-Wallis 1-Way ANOVA). These data suggest that the X<u>Sxr</u> chromosome in XO<u>Sxr</u> mice and the X and Y<u>Sxr</u> chromosomes in XY<u>Sxr</u> are at least partially active transcriptionally. Furthermore, it is possible that the abnormal activity of the sex chromosomes in pachytene spermatocytes of XY<u>Sxr</u> mice was due to the age of the individuals used in the study. Table 8 shows that the average age of the XY<u>Sxr</u> mice were 17.5 months, whereas XO<u>Sxr</u> and XY$^+$ mice were 7.75 and 6.25

months, respectively. However, this conclusion is only speculative since younger XY*Sxr* mice were not available for analysis.

A Spermatid-specific Enzyme. Biochemical differentiation of spermatogenic cells was assessed histochemically. First, the presence of the spermatid-specific LDH isozyme, LDH-C_4, in spermatocytes and spermatids from XO*Sxr* mice was determined by indirect immunofluorescence with antibody for LDH-C_4. Normal XY^+ mice showed a characteristic LDH-C_4 staining pattern in that staining was first detected in primary spermatocytes and increased in intensity through the remainder of meiosis and spermiogenesis. Spermatogonia and Sertoli cells remained unstained. XO*Sxr* and XY*Sxr* spermatids were also positive for LDH-C_4 and exhibited a normal pattern of staining intensity when compared to controls (Fig. 12). Both the large and normal size class of spermatids in XO*Sxr* and XY*Sxr* were brightly fluorescent. These data indicated that there were no temporal or spatial abnormalities in the expression of LDH-C_4, a marker of spermatid differentiation.

Spermatid Nuclear Proteins. Staining with Fast Green and Brilliant Sulfaflavin was used to assess the deposition of basic nuclear proteins in Sertoli cell and spermatogenic cell nuclei of mice bearing the *Sxr* factor. In normal XY mice the nucleolus of Sertoli cells stained deeply with no staining in the remainder of the nucleus (Fig. 13). A progressive increase in staining intensity occurred in nuclei as cells differentiate from spermatogonia to primary

Figure 12. Sections of Seminiferous Tubules Stained by Indirect Immunofluorescence with Rabbit Anti-mouse LDH-C_4.

a. Section from a tubule of an XO<u>Sxr</u> mouse showing a characteristic staining pattern for LDH-C_4. Spermatids (S) stain brightly, whereas spermatocytes (SC) stain less so. Spermatogonia (Sg) remain unstained. X400.

b. Section of a tubule of an XY<u>Sxr</u> mouse. Spermatogonia (Sg) are unstained; spermatocytes (Sc) are lightly stained; and spermatids (S) are brightly stained X400.

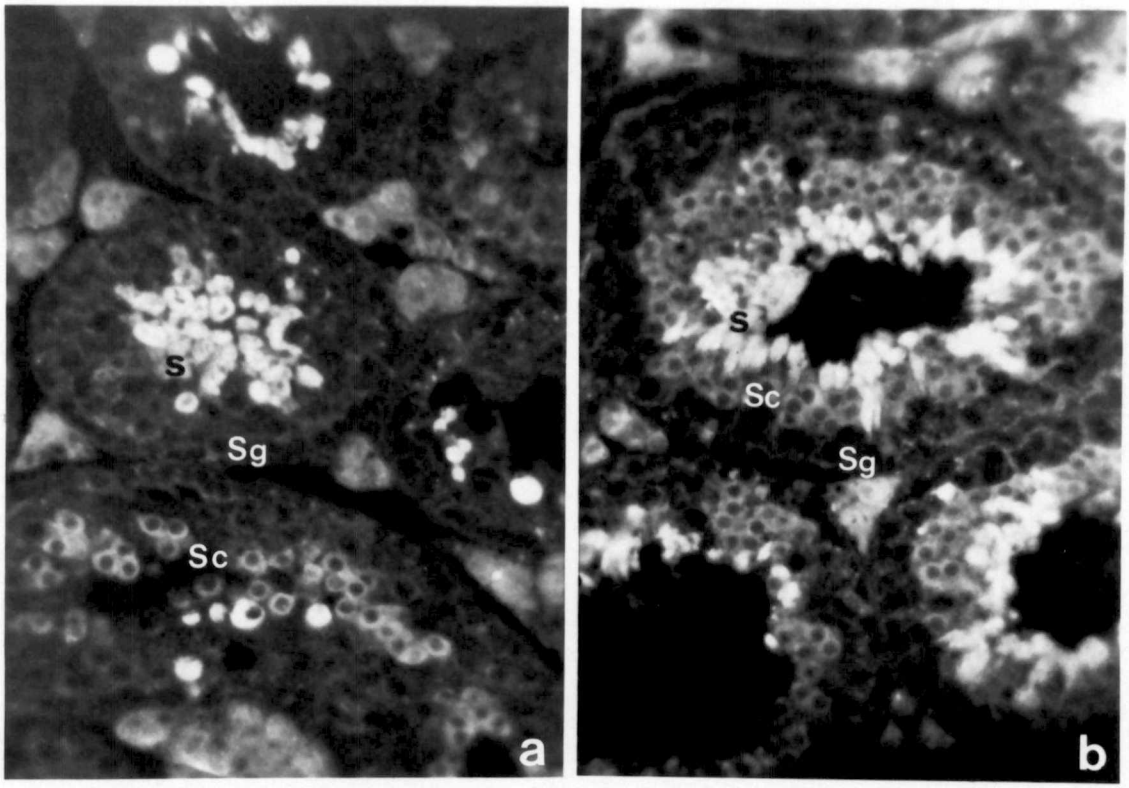
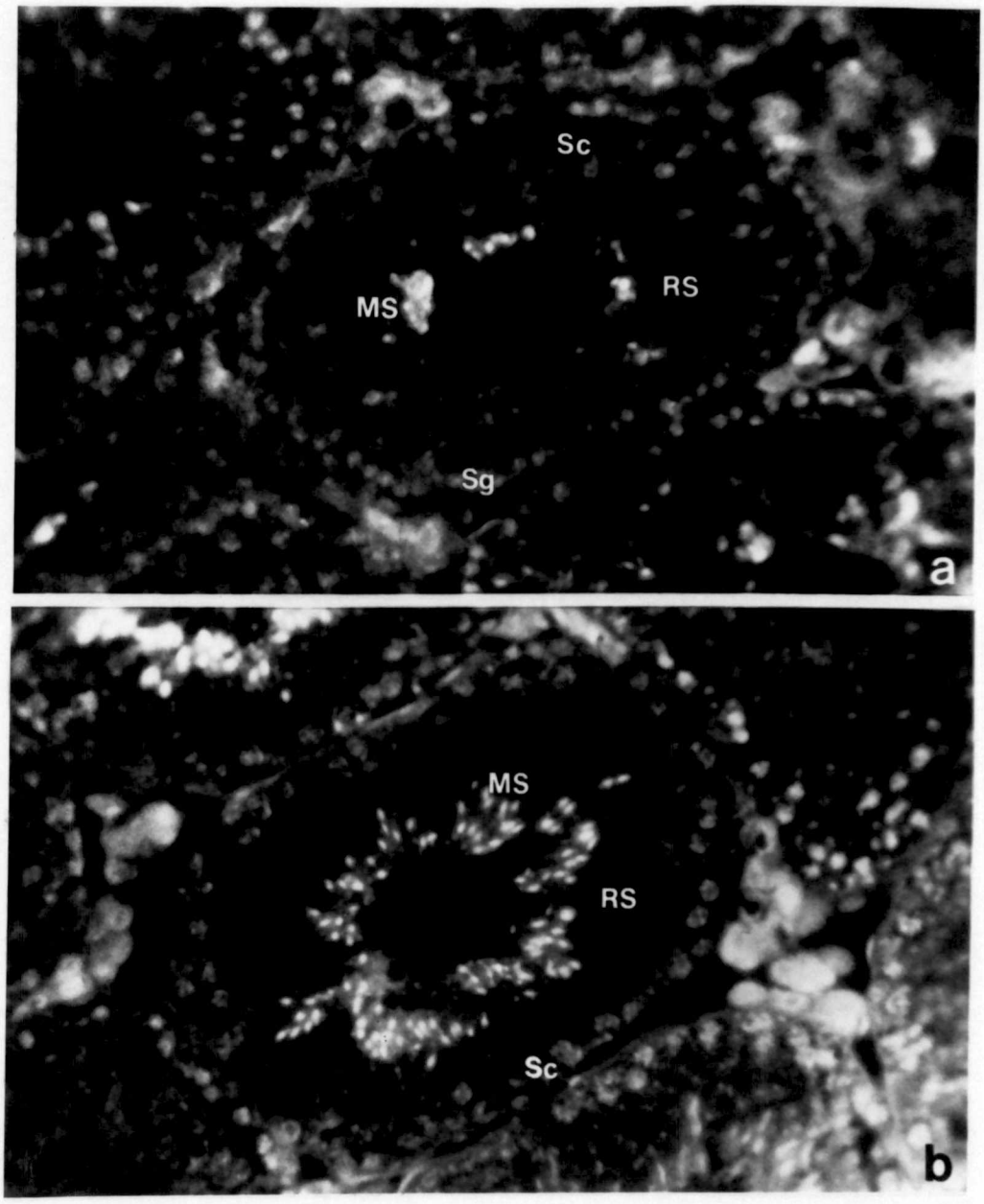

S
Sg
Sc
a
S
Sc
Sg
b

Figure 13. Sections of Seminiferous Tubules Stained with Brilliant Sulfaflavin.

a. Section of a tubule of an XO<u>Sxr</u> mouse showing a characteristic staining pattern for Brilliant Sulfaflavin. Note spermatogonia (Sg), spermatocytes (Sc), and a few spermatids (MS). X450.

b. Section of a tubule of an XY^{+} mouse showing a characteristic staining pattern for Brilliant Sulfaflavin. Basic proteins in spermatogonia and spermatocytes (Sc) stain lightly while round spermatids (RS) do not stain. Mature spermatids (MS) are brightly stained. X450.


Sc
MS
RS
Sg
a
MS
RS
Sc
b

spermatocytes. However, round spermatids did not stain except for lightly staining heterochromatic regions. An increase in staining intensity occurred as cells began the process of elongation and nuclear condensation. Since similar patterns were observed in XO*Sxr* and XY*Sxr* mice (Fig. 13), it appeared that genetic factors necessary for the appropriate temporal and spatial expression of basic nuclear proteins were present in XO*Sxr* mice. Furthermore, sperm head abnormalities observed in XO*Sxr* were probably not due to the absence of basic nuclear proteins.

Function of *Sxr* Spermatozoa

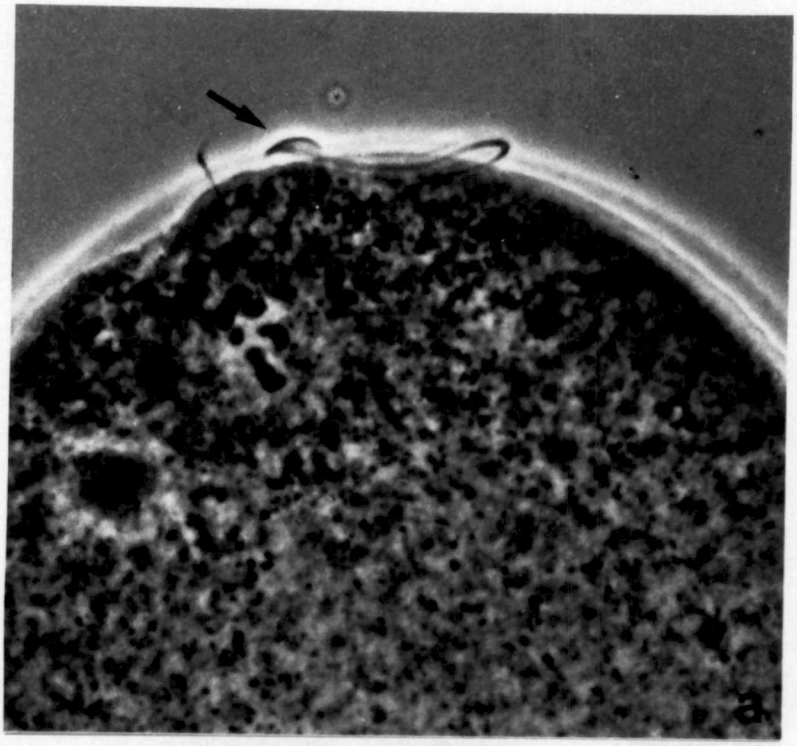
Techniques of *in vitro* fertilization were used to assess the ability of sperm to bind to the zonae pellucidae of mouse eggs (Kot and Handel, 1987). The techniques utilized in these experiments for the removal of sperm from ova resulted in the removal of attached, but not bound sperm.

Sperm from XO*Sxr* mice were able to bind to zonae pellucidae of mouse eggs (Fig. 14), although the sperm bound to fewer eggs and in lower numbers than did sperm of XY*Sxr* or XY^{+} mice. Only 23.2% of the eggs inseminated with sperm from XO*Sxr* mice had sperm bound to zonae while this percentage was statistically higher in eggs incubated with sperm from normal XY mice (68.7%; Student t test, $p<.0001$; Table 9). Sperm from XY*Sxr* mice bound to the zonae pellucidae of mouse eggs in significantly lower numbers, as well; only 37.3% of the eggs had sperm from XY*Sxr* mice bound to them (Student's t test; $p<.0001$). This was

Figure 14. Epididymal Sperm Bound to Zonae Pellucidae.

a. Normal sperm (arrow) from XY^{+} mouse bound to a mouse egg. X485.

b. Type 6 sperm from a XO<u>Sxr</u> mouse bound to a mouse egg. X485.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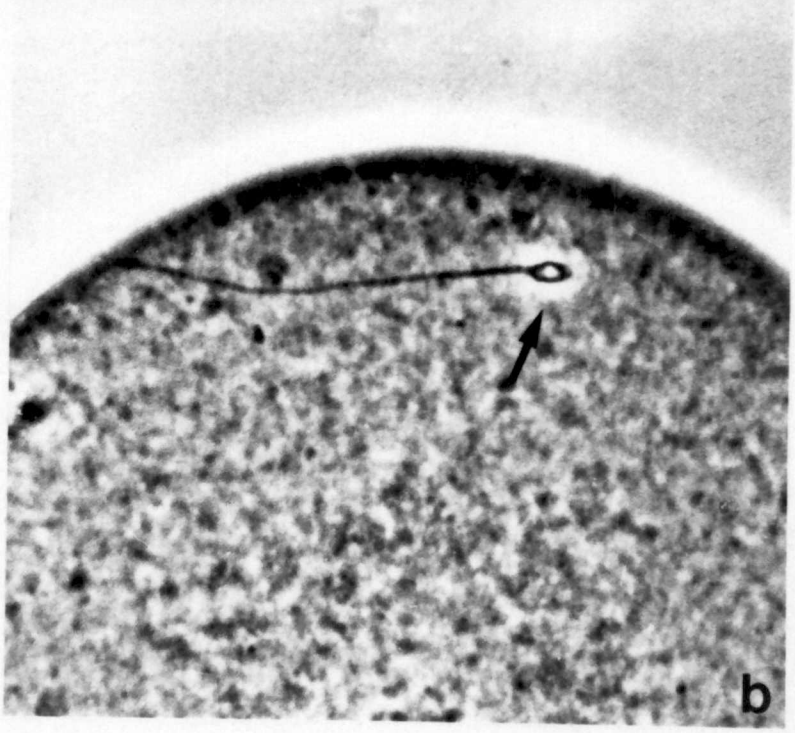
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Table 9. A Comparison of Epididymal Sperm Binding in XO*Sxr*, XY*Sxr*, and XY Mice.

Type of Mouse	Total Number of Ova Scored	Total Number of Ova with Sperm Bound	Percentages of Ova with Sperm Bound	Mean Number of Sperm Per Egg	Percentages of Bound Sperm That Are Abnormal
XY	134	92	68.7	2.37	12.4
XY*Sxr*	67	25	37.3	1.24	35.5
XO*Sxr*	168	39	23.2	1.31	66.7

interesting since XY*Sxr* mice are fertile. However, only older XY*Sxr* mice (8 months or older) were available. Since we have previously demonstrated that significant germ cell reductions occur with age in XY*Sxr* mice it is probable that age is a factor in these results. The mean number of sperm bound per egg varied, as well: XO*Sxr* mice had a mean of 1.31 sperm bound per egg whereas, XY*Sxr* had a mean of 1.24 and XY^+ mice a mean of 2.37 sperm per egg (Table 9). The mean percentage and types of normal and abnormal sperm bound to zonae pellucidae were different in XO*Sxr*, XY*Sxr*, and XY^+ mice. In experiments in which XO*Sxr* sperm were used to inseminate eggs *in vitro*, 66.7% of the sperm bound to the zona were abnormal. Only 12.4% of the bound sperm from normal XY mice were abnormal and 35.5% in XY*Sxr* mice. Four types of abnormal sperm from XO*Sxr* mice bound to zonae pellucidae of mouse eggs: Type 2 (13% of the abnormal sperm bound); Type 3 (3%); Type 5 (37%); and Type 6 (47%). The types of abnormal, bound sperm from XY mice were the same, however, the percentages were different: Type 2 (12%); Type 3 (62%); Type 5 (19%); and Type 6 (6%). The types and percentages of abnormal, bound sperm in XY*Sxr* were Type 2 (27%), Type 3 (18%), Type 5 (9%), and Type 6 (46%). These data demonstrated that sperm from XO*Sxr* mice could bind to the zona pellucida although they did so less efficiently than sperm from normal mice. Finally, since all sperm in XO*Sxr* mice were non-motile, but nonetheless bound to zonae pellucidae, it can be concluded that sperm motility was not essential for zona binding.

IV. DISCUSSION

This study focused on the role of the Y chromosome in germ cell differentiation. Specifically, it involved an investigation of spermatogenesis in the absence of a significant portion of the Y chromosome, with XO*Sxr* mice as the experimental model. Mutations, such as *Sxr*, can provide important information about the control of sexual differentiation and spermatogenesis since they disrupt these processes at specific and different developmental stages. These situations provide ways for analyzing spermatogenic structure and function in the absence of a normal chromosomal constitution.

Sex-reversal in Mammals: Models for Studying Gametogenesis

Successful gametogenesis involves four main steps. The genetic sex of the individual must be established at fertilization by the sex chromosome complement of the zygote. XX and XO mammals are female, whereas XY and XXY mammals are male. Sex determination is then expressed in gonadogenesis, where, in the presence of a Y chromosome, testes develop and, in the absence of a Y chromosome, ovaries develop. The major mammalian sex-determining gene (*Tdy* in mice and *TDF* in humans; Silvers *et al.*, 1982; Page *et al.*, 1987) is thought to be responsible for determination of the testis. Once differentiated, the gonads become essential for further sexual development: development of the sex-specific reproductive tract, secondary sexual characteristics, and gametogenesis. Gametogenesis then proceeds

through the steps of mitotic divisions, meiosis, and the germ cell maturation process, governed by the appropriate controls.

A number of mutations have been reported that disrupt the sequence of events leading to the successful completion of gametogenesis. One category of such mutations causes sex reversal. Sex reversal, or the development of phenotypic characteristics which are contrary to the sex chromosome karyotype of the individual, is known to occur in several species of mammals. Various mutations affecting the sex-determination pathway may be reflected in these conditions. Some mutations probably involve the Y chromosome, while others involve the autosomes or X chromosome. Various sex-reversed conditions differ in the extent of gametogenesis and thus, can be important tools for elucidating the controls of gametogenesis. Nonetheless, similarities exist among some forms of mammalian sex reversal and in the extent of gametogenesis. Sterility and H-Y^+ (a male specific antigen encoded on the Y chromosome) phenotypes are associated with nearly all conditions of XX, sex-reversal (Lyon et al., 1981). Furthermore, human and mouse XX, sex-reversed conditions are almost analogous. Both conditions are characterized by infertility, reduced testicular size, lack of germ cells, and a Sertoli-cell-only syndrome in the adult testes (Cattanach, 1975; de la Chapelle, 1972; Weger and Numberger, 1983). The genetic mechanisms of sex-reversal in XX human males and Sxr mice are also similar; both are caused by the presence of Y-chromosome material in XX males (Evans et al., 1982; Page et al., 1987; Singh and Jones, 1982).

However, there are also differences in both the extent of gametogenesis and the genetic mechanisms that cause sex reversal among different species. In contrast to the sex reversal in the mouse, which is caused by Sxr mutation, sex reversal in the goat is caused by an autosomal gene, Polled (P; Hamerton et al., 1969), and is induced in individuals homozygous for that gene. There is no known evidence for the presence of any Y-chromosomal material in these males. Masculinization is incomplete in sex-reversed goats since intersexes are produced (Watchel, 1983). These intersexes may possess ovaries, testes, or ovotestes (Cattanach, 1975). As in XXSxr mice, germ cells are present in the sex-reversed, fetal testes of goats, but are usually absent in the adult testes, causing a Sertoli cell-only condition (Cattanach, 1975). This is an interesting situation since testes, Sertoli cells, and, occasionally, germ cells develop in the apparent absence of the Y chromosome. In pigs, sex reversal is poorly understood but is thought to involve environmental, as well as genetic factors (Lyon et al., 1981). XX male gonads are often ovotestes (Cattanach, 1975). Germ cells are not present within the testes or testicular portion of the ovotestes but are present in the ovarian portion of the ovotestes (Cattanach, 1975). The exact role of the Y chromosome in these forms of sex-reversal, if there is any at all, is unknown. These studies suggest that, while the Y chromosome has an important role in testis determination, autosomal and X-linked factors, as well as environmental cues, are also involved. However,

these studies provide little information about the controls of spermatogenesis.

Although there are a number of situations involving females sex-reversed to males, XY females can also be associated with the sex reversal phenomenon. XY females have been described in wood lemmings, cattle, horses, dogs, rats, mice, and humans (Lyon et al., 1981). Human XY females are characterized by a normal external female phenotype, gonads which are malformed and lack germ cells, and often, the development of gonadoblastomas in one or both gonads (Page, 1986). Most human XY females have a small deletion in the Y chromosome in the region of TDF, whose size varies (Page, et al., 1987).

In the mouse, a mutation, testicular feminization (Tfm), causes another form of sex-reversal. Tfm individuals are characterized by undescended testes, an external female phenotype, and high levels of circulating testosterone (Wilson et al., 1983). The defect is due to a deficiency of normal androgen receptors in the target tissues, resulting from a mutation in an X-linked gene (Migeon et al., 1981). Therefore, both X-linked and Y-linked genes are necessary for the normal differentiation of a testis. Similar mutations have been described in humans, rats, dogs, and chimpanzees (Wilson et al., 1983).

Certainly, then, more detailed studies of sex-reversed mammals can further our understanding of mammalian gametogenesis. It is clear that the study of different kinds of sex reversal can provide different kinds of insights into the controls of spermatogenesis.

Past studies have already demonstrated that autosomal and X-linked genes are necessary for sex-determination and spermatogenesis. For example, by studying the Tfm mutation, investigators have learned that a receptor is required for normal testosterone action in males and females and is X-linked. Furthermore, these conditions have shown that two X chromosomes are detrimental to germ cell differentiation in a testis. However, these studies have failed to identify any Y-linked factors involved directly in the controls of spermatogenesis.

In this study Sxr mice were exploited to investigate the role of the Y chromosome in spermatogenesis. Several experimental questions were addressed. First, are Sertoli cells morphologically normal in mice carrying SXr fragment? If not, do the anomalous conditions provide clues to the controls of Sertoli cell differentiation? Second, do spermatids of these mice lack any specific structures that may be encoded or controlled by a gene(s) in the portion of the Y chromosome that is missing in these mice? Third, is spermatogenesis quantitatively normal in XOSxr mice, and if not, does the first appearance of abnormalities provide any clues about the genic or chromosomal function of the Y chromosome? Fourth, are the spermatozoa of XOSxr mice morphologically normal and, if not, is there evidence for genes controlling sperm morphology on the Y chromosome? Fifth, do spermatozoa of XOSxr mice function in various aspects of fertilization? Can any abnormalities in the function be related to the missing portion of the Y chromosome in XOSxr mice? Since little is known about the causes of male sterility, these studies can begin

to determine if male sterility is a consequence of structural abnormalities of spermatogenic cells, of abnormal or missing Y-linked products, or of abnormal chromosomal behavior. These studies should, then, provide insights into some of the causes of male sterility.

The present study has provided data concerning the morphology of Sertoli cells, sperm-specific structures, the kinetics of spermatogenesis, and spermatid size in Sxr mice. In addition, this study provided information about the morphological and functional aspects of spermatozoa in these mice.

Sertoli Cells in Sxr Mice

Ultrastructural analysis revealed structurally normal Sertoli cells within the seminiferous epithelium of both XOSxr and XYSxr mice. This demonstrates that the gene(s) necessary for Sertoli cell differentiation is present in XOSxr mice. The gene(s) is probably located on the Y chromosome, and thus, in the Sxr segment, since Burgoyne et al. (1988) showed that the Y chromosome is required for Sertoli cell development. They analyzed XX<—>XY chimeric mice (by isozyme and DNA markers) to determine if both components can contribute to the Sertoli cell population. The finding was that the Sertoli cells were exclusively of the XY component of the chimera. They suggested that the Tdy gene acts to initiate Sertoli cell differentiation and, subsequently, testis formation. Recent findings (Guttenbach et al., 1989) seem to confirm the hypothesis that genes responsible for Sertoli cell differentiation are located on the Y

chromosome. Hybridization of two Y-linked probes in situ to Sertoli and somatic cells of adult mice was used to investigate the conformational state of Y-chromosome DNA. Unlike the Y chromosome in various somatic cells, the Y chromosome of the Sertoli cell was always in a highly decondensed state, suggesting the possibility of continued transcriptional activity even after determination of Sertoli cells. Therefore, the findings of Guttenbach et al. (1989) and Burgoyne et al (1988) support the view that the Y chromosome is responsible for both Sertoli cell differentiation and continued function. Since XOSxr mice have morphologically normal Sertoli cells, they must have the Y-linked gene(s) responsible for initial Sertoli cell differentiation.

It is interesting to note that the Sertoli cells of XOSxr mice were normal, while those of XXSxr mice were abnormal. Both kinds of mice have equivalent segments of the Y chromosome but they differ in the number of X chromosomes. Furthermore, adult XOSxr mice have germ cells present in their seminiferous tubules, whereas XXSxr testes are devoid of germ cells. Chung (1975) suggested that the ultrastructural abnormalities of the Sertoli cells in XXSxr mice result from the degeneration or absence of germinal epithelium. However, Handel and Eppig (1979) showed that germ cells are not a prerequisite for the postnatal, prepubertal differentiation of Sertoli cells. They used two mutations of the mouse that cause germ-cell deficiency to show that normal ultrastructural differentiation of Sertoli cells occurred in the absence of germ cells. Thus, maturing germ cells are not necessary for the ultrastructural differentiation of Sertoli cells.

However, even though germ cells are not essential for ultrastructural differentiation of Sertoli cells, their function and long-term maintenance in the adult may depend on signals received from maturing germ cells. Therefore, the morphological abnormalities observed in Sertoli cells of XXSxr mice may not be due to the absence of germ cells, but instead, to the presence of other factors in these mice.

Another possible cause for Sertoli cell abnormalities in XXSxr mice may be that the presence of two X chromosomes is somehow detrimental to the normal development of Sertoli cells. In normal XY, XOSxr, and XYSxr males only one X chromosome is present. These mice all exhibit morphologically normal Sertoli cells. XXSxr mice have two X chromosomes and morphologically abnormal Sertoli cells. In addition, Lifschytz and Lindsley (1972) have proposed that inactivation of a single-X chromosome of primary spermatocytes is essential for normal spermatogenesis, suggesting that excessive X chromosome product(s) is not conducive to normal development of male-specific cells. Further, a small number of XXSxr germ cells enter prophase of meiosis I before birth and develop as presumptive spermatogonia (McLaren, 1981). These germ cells have higher hypoxanthine phosphoribosyl transferase (HPRT) activity than that of spermatogonia in normal XY mice (McLaren and Monk, 1981). Since HPRT is an X-linked gene, this increased activity was thought to be associated with a reactivation of a silent X chromosome (McLaren and Monk, 1981). It may be that a similar event, which supports the transcriptional activity of two X chromosomes, operates in Sertoli

cells of XXSxr mice, as well. These hypotheses and findings suggest that two X chromosomes may be detrimental to the normal morphological development of Sertoli cells in XXSxr mice.

In addition to Sertoli cells within the seminiferous epithelium of XXSxr mice, another somatic cell was found; anomalous, "Type 2" somatic cells were present in clusters. Chung (1975) suggested that these cells were immature, abnormal Sertoli cells. However, they are also morphologically similar to macrophages, which are characterized by a round nucleus containing large areas of heterochromatin and nuclear indentations and large numbers of lysosomes and pinocytotic vesicles (Junqueira and Carneiro, 1980). Since these features are characteristic of anomalous, Type 2 somatic cells of the seminiferous tubules of XXSxr mice, these cells may, in fact, be macrophages. Interestingly, these cells were usually observed in disturbed tubules which appeared to have insufficient Sertoli-cell junctions, thus, possibly allowing for invasion of macrophages. Fritz et al. (1983) reported that the Sertoli-cell junctions of XXSxr mice were not competent as tight junctions. They analyzed the distribution of [^{3}H]-sucrose and [^{14}C]-urea in the testes of normal and XXSxr mice and found defective seminiferous tubule barriers. Thus, their data support the view that the Sertoli-cell junctions of XXSxr mice may allow the invasion of macrophages. Definitive resolution of the identity of these cells as macrophages will depend on the detection of specific cell-surface markers unique to macrophages.

Sperm-specific Structures

The results of this study indicated that pachytene spermatocytes from XO*Sxr* mice were able to form the sex vesicle, a structure unique to spermatogenic meiocytes. This confirmed a similar observation by Cattanach *et al.* (1971). It is interesting to note that even in the absence of a Y chromosome, synaptonemal complex was observed within these sex vesicles. This may be a reflection of fold-back pairing of the X*Sxr* chromosome or the anomalous synaptonemal complex of the X chromosome found in normal XY mice (Solari, 1970). Thus, these data imply that an intact Y chromosome and normal X-Y pairing are not essential for the formation of the sex vesicle. The elaboration of this structure, then, may depend only on the presence of an X chromosome, on chromosome condensation, on genes in the small portion of the Y chromosome present within the *Sxr* segment, or on other, unknown factors.

The results of this study demonstrated that all sperm-specific structures and events (such as acrosome development, manchette differentiation, nuclear condensation and elongation, and tail formation) and at least two sperm-specific proteins (LDH-C_4 and basic nuclear proteins) were present and elaborated in the correct temporal and spatial pattern in spermatids of XO*Sxr* mice. These observations provide proof that the genes encoding these structures are not located within the portion of the Y chromosome that is lacking in XO*Sxr* mice. Therefore, the genes involved must be on autosomes, on the X chromosome, or within the *Sxr* fragment.

The presence of genes on the Y chromosome that control spermatogenesis has long been postulated. In humans, deletions of the Y chromosome beyond the distal portion of band q11 have been associated with azoospermia and, often with hypogonadism (Alvesalo and de la Chapelle, 1981; Neu et al., 1973; Tiepolo and Zuffardi, 1976; Yunis et al., 1977). In addition, Chandley and Edmond (1971) reported an azoospermic patient whose Y chromosome was in a ring conformation. Individuals with a ring-Y chromosome probably have lost the fluorescent portion the Y chromosome, and possibly some of Yq (Stewart, 1983). Thus, these data suggest, but do not confirm, that factors on the long arm of the human Y chromosome may control spermatogenesis. From these observations one might conclude that there could be genes involved in spermatogenesis on the mouse Y chromosome. However, the data presented here indicate that there are no genes controlling the initiation or temporal pattern of spermatogenesis, or the sperm-specific structures on the major part of the mouse Y chromosome. It is ,then, possible that such genes are within the Sxr region. This segment is extremely small cytologically, but large genetically. Goodfellow and Darling (1988) suggested that Sxr is about $5X10^6$ bp in size (determined cytologically), or approximately 5cM. To illustrate, the "t complex" on chromosome 17, a region including the T (Brachyury) locus through the major histocompatibility complex, is approximately 12-15cM, or 1% of the mouse genome (Silvers, 1985). Therefore, the Sxr fragment could encompass a large number of genes and include all genes for sperm-

specific structures. However, the existence of specific genes on the Y chromosome that affect spermatogenesis, either directly or indirectly, must still be determined. Furthermore, while it is possible that the Sxr fragment contains the genes that control sperm cell differentiation, it also possible, and perhaps even more likely, that these genes are encoded on autosomes and/or on the X chromosome. All presently known genes affecting spermatogenesis or sperm-specific products have been mapped to various autosomes (see Handel, 1987 for review). For example, the gene for a sperm-specific enzyme, phosphoglycerate kinase-2 (PGK-2), has been assigned to chromosome 17 (Eicher et al., 1978), and the genes for sperm-specific protamines have been mapped to chromosome 16 (Hecht et al., 1986). Therefore, it would not be al all surprising if genes for sperm-specific structures, such as the acrosome or tail components, also were on autosomal chromosomes.

Even though spermatids of XOSxr mice assembled specific components in a temporally and spatially normal fashion, ultrastructural analyses also revealed a high frequency of morphological abnormalities of spermatids in XOSxr and XYSxr mice. These anomalies included multiple proacrosomal vesicles, double or bifurcated heads, and aberrant head shapes. Such abnormalities are not unique to Sxr mice. For example, the mutation pink-eyed sterile (p^{6H}) causes formation of spermatids with multiple proacrosomal vesicles (Hunt and Johnson, 1971; Bryan, 1977), similar to those described here for XOSxr mice. Thus, the morphological abnormalities

described for XO<u>Sxr</u> mice may not be due to the direct action of one or more genes, but rather to disturbed cell interactions within the testis, particularly between Sertoli cells and germ cells. Sertoli cells show seminiferous tubule-stage specificity in the synthesis and secretion of a variety of factors. It is reasonable to suppose that the synthesis and release of these factors from Sertoli cells is controlled by signals received from germ cells in various stages of differentiation (Griswold, 1988). An absence, or even a reduction, in these signals, due to decreased numbers of differentiating germ cells, could render Sertoli cells unable to produce factors needed for germ-cell differentiation (see discussion in Handel, 1987). Since meiotic and post-meiotic cells are significantly reduced in XO<u>Sxr</u> mice, there may be inadequate numbers of these germ cells to produce sufficient quantities of those signals that induce Sertoli cell synthesis and secretory action. Any major perturbation of spermatogenesis resulting in decreased numbers of germ cells might, then, indirectly initiate, through Sertoli cells, a wide array of structural and/or physiological abnormalities in developing germ cells. Thus, it is proposed that the initial defect causing reduced numbers of spermatocytes and spermatids may be amplified when Sertoli cells fail (in the absence of adequate signaling from normal numbers of germ cells) to produce the appropriate microenvironment for normal differentiation of spermatids. This cascade of events may be the correct explanation for the morphological abnormalities of spermatids of XO<u>Sxr</u> mice, since it has been previously demonstrated that testes with low numbers of germ

cells produce morphologically abnormal spermatids (Handel, 1987; Handel et al., 1987).

Kinetics of Spermatogenesis

Significant depletion of spermatogenic cells was observed in XOSxr and XYSxr mice. While numbers of spermatogonia were not significantly reduced in these mice, most meiotic cell types and all post-meiotic cell types were reduced in number relative to Sertoli cells. Reductions in numbers of spermatogenic cells, according to these results, apparently began at the onset of meiosis and continued throughout meiosis and spermiogenesis. These results differ from those of Hannappel and Drews (1979) and Burgoyne and Baker (1984) who reported a reduction in spermatocyte number as beginning at pachynema, rather than at the onset of meiotic prophase. However, since they evaluated only stage VII and XII tubules, they thereby excluded early meiotic cells (for example, leptotene and zygotene spermatocytes) for analysis and cannot draw conclusions relative to these cells. In the present study, morphologically abnormal pachytene spermatocytes were occasionally observed in the seminiferous tubules from XOSxr mice. However, their numbers were small and could not alone account for the reduction in numbers of pachytene spermatocytes.

In addition, this study revealed that numbers of spermiogenic cells were dramatically and significantly reduced in XOSxr and XYSxr mice. These reductions were observed from the onset of spermiogenesis and were even greater than the reduction in number of meiotic cells.

Theoretically, if germ cell loss were due to the atresia of cells during meiosis I, then spermatids should be reduced by a factor of approximately four times from the expected number of post-meiotic cells. However, there was a twelve-fold reduction from the expected in XO*Sxr* and an approximately nine-fold reduction in XY*Sxr* mice. In addition, in XO*Sxr* mice there were approximately 0.5 times as many spermatids as spermatocytes, instead of the expected four times as many spermatids as spermatocytes. Cattanach *et al.* (1971) suggested that reduced numbers of spermatids in XO*Sxr* mice indicated failure in meiotic divisions and general meiotic disturbance. Burgoyne and Baker (1984) and Levy and Burgoyne (1986) proposed that most of the spermatids in XO*Sxr* mice (up to 92%) omit meiosis II. The present results suggest that this is a likely mechanism for reduction in numbers of spermatids in XO*Sxr* and XY*Sxr* mice. However, there is approximately a four-fold reduction from the expected in the number of cells entering spermiogenesis and elimination of a meiotic division would account for only a two-fold reduction. Therefore, in addition to meiotic division failure, other factors must be involved in spermatid loss.

Even though the exact cause of germ cell loss in XO*Sxr* and XX*Sxr* mice is unknown, at least four mechanisms, which are not necessarily mutually exclusive, can be postulated. First, it is possible that the *Sxr*, or Y transposition, on the X chromosome or the Y chromosome may somehow interfere with the normal process of meiosis. For example, the presence of extra sex-chromosomal material may disrupt the pairing

process between the X and Y chromosomes which occurs during prophase I of meiosis I and lead to abnormal meiosis in XY<u>Sxr</u> mice. In the case of XO<u>Sxr</u> mice, it is possible that the presence of a single sex chromosome, without a pairing partner, is inhibitory to meiosis. In fact, Miklos (1974) has proposed that failure to completely saturate all pairing sites leads to spermatogenic failure. The failure proposed as a result of this mechanism does not necessarily occur during meiosis. In XYY mice, the presence of two Y chromosomes impedes germ cell differentiation at the time of meiosis. This suggested to Burgoyne (1979) and Burgoyne and Biddle (1980) that abnormalities in XYY mice resulted from failure in the pairing process, and unsaturated pairing sites among the three sex chromosomes. Likewise, in XO<u>Sxr</u> mice, the entire pairing region of the X chromosome may be "unsaturated", and in XY<u>Sxr</u> mice, the <u>Sxr</u> region adjacent to the pairing region, may be "unsaturated" (i.e., without a paring partner). However, it is difficult to understand exactly how "unsaturated" pairing sites could lead to alterations of spermatogenesis (see Handel, 1987 for discussion). It is not clear exactly what a pairing site is, how the cell identifies it, or how the cell determines saturation. It is also difficult to accept this theory as a sole explanation of germ cell loss during spermatogenesis in XO<u>Sxr</u> and XY<u>Sxr</u> mice, since we observed significant reductions in cell number prior to the onset of sex chromosome pairing (presumed to occur at pachytene of meiosis I; Solari, 1974). Thus, while the presence of the <u>Sxr</u> region may impede pairing and, therefore, disrupt

spermatogenesis, this cannot be the only, or even the first, cause of germ cell death during meiosis in XO<u>Sxr</u> mice.

Second, the reduction in numbers of meiotic cells of XO<u>Sxr</u> and XY<u>Sxr</u> mice may be due to improper function of the X chromosome, normally inactivated during male meiosis. Lifschytz and Lindsley (1972) proposed that translocations involving the X somehow interfere with inactivation of the X chromosome during meiosis and that inactivation of the X chromosome is an essential event of spermatogenesis. Forejt (1982) added to this theory by suggesting that X chromosome products are detrimental to male meiosis and that failure to inactivate the X chromosome during meiosis leads to spermatogenic failure. However, a mechanism for this has not been proposed. Since it was found here that pachytene spermatocytes of XO<u>Sxr</u> mice had significant incorporation of [^{3}H]-uridine into the sex vesicle, it appears that failure, or incomplete X-chromosome, inactivation occurred. This might contribute, in an unknown way (for instance, by inhibition of meiosis by an X-encoded protein), to the loss of meiotic cells observed in XO<u>Sxr</u> mice. If improper X-inactivation is the cause of meiotic germ cell loss, then X-chromosome inactivation may begin before, or at, the onset of meiosis, since preleptotene spermatocytes were significantly reduced in number. Since it is possible that incorporation of [^{3}H]-uridine into the sex vesicles in pachytene spermatocytes of XO<u>Sxr</u> mice reflected incomplete inactivation of the X<u>Sxr</u> chromosome, further experimentation involving analyses of specific X-linked probes is necessary to confirm this

interpretation and to determine the extent of X-inactivation in XO*Sxr* mice.

Third, an equally valid interpretation of results from the transcriptional studies utilizing [^{3}H]-uridine labeling, followed by autoradiography, is that the *Sxr* segment, not the X chromosome, is transcriptionally active within the sex vesicle of XO*Sxr* and XY*Sxr* mice. In normal XY mice both neither the X or Y chromosome in pachytene spermatocytes incorporate [^{3}H]-uridine (Kierszenbaum and Tres, 1974 a,b; Monesi *et al.*, 1978). Therefore, since both the X chromosome and the *Sxr* fragment are present within the sex vesicle of XO*Sxr* mice (and an X and Y chromosome and the *Sxr* fragment, in XY*Sxr* mice), it is possible, if not likely, that the *Sxr* fragment is transcriptionally active and interferes with the normal events of meiosis (perhaps by producing a protein detrimental to meiotic cell differentiation, for example). However, a mechanism for this action has not been demonstrated, nor is it understood how transcriptional activity of the *Sxr* fragment could disrupt meiosis. Further study is needed to determine: 1) if X chromosome, the *Sxr* fragment, or both, is transcriptionally active in pachytene spermatocytes (by utilizing *in situ* hybridization of X and Y-linked probes to pachytene spermatocytes); 2) how improper sex-chromosome inactivation could disrupt meiosis; and 3) what specific products, synthesized during meiosis I, are detrimental to developing germ cells.

A fourth possible cause for abnormalities in the kinetics of spermatogenesis in XO*Sxr* could be the lack, or reduction, of essential

Y-encoded gene products affecting both spermatocytes and spermatids. It is possible that such products could be growth factors or meiotic inducers. Such gene products may be required for the transition of cells from mitosis to meiosis and from meiosis to spermiogenesis. If such positive controls exist and are encoded on the Y chromosome, then spermatocytes from these animals would be unable to enter a new differentiative pathway. Germ cells from XO*Sxr* and XY*Sxr* mice do seem unable to embark efficiently on new differentiative pathways, since major reductions in germ cell numbers occur at the onset of any new stage in the spermatogenic process (for example, at meiosis and spermiogenesis). If the lack of Y-encoded products is responsible for the reduction of germ cells in XO*Sxr* mice, then it is not clear why some cells are able to diffferentiate, albeit often abnormally, while others degenerate.

Finally, the decrease in both spermatocytes and spermatids in XO*Sxr* and XY*Sxr* mice may be due to an improper testicular microenvironment. Sertoli cell junctions create two compartments within a seminiferous tubule; the basal compartment contains spermatogonia and preleptotene spermatocytes, while the adluminal compartment contains meiotic and post-meiotic cells. Each compartment is separated from the other by the Sertoli cell junctions, thus allowing two different microenvironments to be present. It has been postulated that unique Sertoli cell-germ cell interactions occur in a stage-specific manner (La Croix *et al.*, 1981; Ritzen *et al.*, 1982; Wright *et al.*, 1983, Griswold, 1988). Sertoli cells are thought to

provide various structural and biochemical factors, necessary for sperm cell differentiation, which are produced and/or released under the control of signals manufactured by the developing germ cells (Griswold, 1988). Handel (1987) has suggested that reciprocal Sertoli cell-germ cell interactions may be required to produce a microenvironment conducive to normal germ cell differentiation and that inadequate numbers of developing germ cells may be unable to trigger Sertoli cell production of appropriate structural and biochemical factors required for continued spermatogenesis. Therefore, the initial reductions in the numbers of germ cells in XO*Sxr* and XY*Sxr* mice may cause a cascade of events. Sertoli cells may receive inadequate signals from the germ cell, causing the Sertoli cells to synthesize inappropriate amounts of essential products. The lack of critical Sertoli-cell products, then could cause a reduction in germ cells. This hypothesis may, at least in part, account for the reduced number of germ cells, especially late spermatids, in XO*Sxr* and XY*Sxr* mice.

Spermatid Size

Two populations of spermatids from XO*Sxr* and XY*Sxr* mice were described: large and normal-sized. Both were found at every step of spermiogenesis, and the percentage of each remained constant throughout spermatid differentiation for a given genotype. This observation indicates that the large-size spermatids were not preferentially degraded. Hannappel and Drews (1979), Hannappel *et al.*

(1980), Burgoyne and Baker (1984), and Levy and Burgoyne (1986) also described large, round spermatids in Sxr mice. Hannappel and Drews (1979) reported that large spermatids of XYSxr mice were first observed among Golgi-stage round spermatids, but were not found beyond the cap-phase of round spermatids in tubules from XYSxr mice. The data reported here for XOSxr and XYSxr mice demonstrate that large spermatids were found throughout spermiogenesis.

Levy and Burgoyne (1986) suggested that the presence of large spermatids was not a direct effect of the Sxr factor, but a more general manifestation of disturbed spermatogenesis. The presence of large spermatids within the seminiferous tubules is not unique to XOSxr and XYSxr mice. Such cells have been observed in the seminiferous tubules of mice heterozygous for the T(5;12)31H translocation (Levy and Burgoyne, 1986), the T(6;12)32H translocation (de Boer et al, 1986), and in Ts(1^{13})70H mice (de Boer et al., 1986). Since large-sized spermatids are not a unique manifestation of the Sxr condition, several factors could contribute to the production of spermatogenic abnormalities. First, abnormalities in pairing could cause spermatocytes to omit a meiotic division. Burgoyne et al (1985), Burgoyne and Baker (1984), Levy and Burgoyne (1986) suggested that large-sized spermatids were diploid and resulted when meiosis II was omitted by spermatogenic cells. These authors find a reduction in the number of meiotic II figures compared to normal numbers of meiotic I figures in the testes of XOSxr mice. Here, however, MI and MII figures were reduced, but not in comparison to the other. In

addition, Burgoyne and Baker (1984) suggested that large-sized spermatids in XYSxr mice resulted from spermatocytes that undergo X-Y chromosome pairing failure. While abnormalities ensuing from sex-chromosome pairing failure may result in the production of some large-sized spermatids in XOSxr and XYSxr mice, this is unlikely to account for the total number of abnormally large-sized spermatids. Second, improper sex-chromosome inactivation may also be indirectly responsible for the generation of abnormally large round spermatids by altering the normal gene expression and the differentiative events of meiosis. A protein, not normally present during meiosis and encoded on the X or Sxr fragment, could cause the production of abnormal spermatocytes and, eventually, large-sized spermatids. The exact protein, if any at all, and associated mechanism for this type of action are, however, unknown. Third, reduced numbers of germ cells could also account for presence of the large-sized spermatids in XOSxr and XYSxr mice by creating an improper testicular microenvironment in association with the Sertoli cells (see page 77). Any, or all, of these factors could contribute to the production of large-size spermatids observed in XOSxr and XYSxr mice.

Morphology and Function of Spermatozoa from Sxr Mice

Morphological studies revealed that XOSxr and XYSxr mice exhibited wide variability in both the percentage and types of abnormal epididymal sperm. These abnormalities were not unique to the Sxr condition, and so may be due to a disturbed testicular environment

where insufficient numbers of germ cells are unable to adequately communicate with the Sertoli cells (see page 77). The observed variability between mice may be due to the presence of different sized fragments of the Sxr region within each XOSxr and XYSxr mouse. This variability could be produced during pachytene of meiosis I in the carrier, XYSxr males when the Sxr region is transferred from the Y to the X chromosome during a cross-over event. If cross-over can occur at any of various points along the Sxr region of the Y chromosome, then slightly different XSxr chromosomes would be produced. This could result in Sxr fragments of various lengths of and ultimately in the presence or absence of different genes in different individuals. This variation in the Y-linked Sxr material may account for the differences observed in XOSxr and XYSxr epididymal sperm between individuals. McLaren (1988) has suggested a similar phenomenon to account for differences in the Sxr region between Sxr and Sxr' mice. She postulated that Sxr' involved a small deletion, brought about by an unequal cross-over. If, in fact, the variation that was observed in the numbers and types of sperm abnormalities is due to differences in the size of the Sxr fragment, then factors controlling sperm morphology may be present on the Y chromosome, as suggested by the work of Krzanowska (1971, 1976) and Styrna et al. (1989). Another interpretation of the variability in the percentages and types of abnormal sperm between individuals could be that differences in the size of the Sxr fragment, if they exist, cause different extents of

abnormal chromosome behavior. For example, the number of "unsaturated"-pairing sites would be different in Sxr mice with different lengths of the Sxr fragment. Miklos (1974) suggests that unsaturated pairing sites could cause abnormalities in spermatogenic cell differentiation which would not have to be confined to meiosis. It is conceivable that an increase in unsaturated pairing sites could cause an increase in abnormalities. Additional X chromosome or Sxr product could be produced in animals with larger sizes of the Sxr fragment since the XSxr fragment is transcriptionally active in XOSxr mice. Therefore, differences in the length of the Sxr fragment among individuals could cause differences in the pairing process which, in turn, would produce differences in the percentages and type of sperm abnormalities. This seems a more plausible interpretation than that of morphological factors being encoded on the Y chromosome since no such factors have been identified.

Functional aspects of XOSxr sperm were assessed by zona-binding studies demonstrating that sperm from XOSxr mice could bind to zonae pellucidae of mouse eggs, although they did so in much lower numbers than did sperm from normal mice. The zona pellucida, which is a glycoprotein coat that surrounds the egg, provides species specificity to the fertilization process. This specificity is due to the presence of specific receptors that recognize ligands on the sperm head. The zona proteins ZP1, ZP2, and ZP3 (Wasserman,1987a,b) must be present for the sperm to bind to, and ultimately penetrate, the egg. The data from the current study indicate that the sperm ligands necessary for

zona binding were present on sperm from XO*Sxr* mice and, therefore, cannot be encoded within the portion of the Y chromosome missing in these mice. However, there was reduced efficiency of binding in sperm of XO*Sxr* mice which may have been caused by several factors. Kot and Handel (1987) showed that abnormal sperm bound *in vitro* to zonae pellucidae less frequently than did normal sperm and that sperm from strains of mice which were characterized by high percentages of abnormal sperm bound to significantly fewer eggs. A number of investigators have suggested that sperm motility is an essential factor in the penetration of the zona pellucidae (Fraser, 1981; Olds-Clarke, 1983; Tessler *et al.*, 1983; and Yanagamachi, 1981). An accelerated, whiplash, flagellar beating (hyperactivation) may cause the initiation of the acrosome reaction and provide the thrust needed to propel the sperm through the zona pellucida. Therefore, the reduced efficiency of binding by sperm from XO*Sxr* mice could be due to the absence of sperm motility, to extremely high percentages of abnormal sperm, to greatly reduced sperm numbers, or to other morphological or physiological abnormalities.

In vivo sterility of XO*Sxr* mice may be due to the reduced number of epididymal sperm. It has been observed that a sperm count 20% of normal is compatible with fertility, but a sperm count less than 20% of normal is associated with sterility. In the current study, XO*Sxr* mice were found to have a sperm count approximately 10% that of normal XY mice. Since the numbers of epididymal sperm are, thus, dramatically reduced in XO*Sxr* mice, it is resasonable to propose that

sterility in XO<u>Sxr</u> is due, wholly or in part, to reduced numbers of epididymal sperm.

Sperm from XO<u>Sxr</u> mice were not motile, whereas sperm from normal and XY<u>Sxr</u> mice did exhibit motility. Thus, it cannot be ruled out that the portion of the Y chromosome that XO<u>Sxr</u> mice lack may encode genes necessary for sperm motility. This agrees with an hypothesis put forth by Eicher <u>et al</u>. (1983) based on cytogenetic and molecular studies of two mouse mutants with rearranged Y chromosomes, XY<u>Sxr</u> and Y^*, in addition to XO<u>Sxr</u> mice. Y^* is a rearranged Y chromosome. When an XY^* male is mated to an XO female, a recombinant X^YO male is produced. These males have a large part of the Y chromosome attached to their paternal X. X^YO mice produce numbers of mature, motile sperm, unlike XO<u>Sxr</u> mice which produce non-motile sperm. Eicher <u>et al.</u> suggested that the gene(s) necessary for sperm motility are present in X^YO males, but not in XO<u>Sxr</u> males. If this is the case, then the gene(s) must be in a region of the Y chromosome between the centromere and the pairing region that is not present in XO<u>Sxr</u> mice. The results of the current study are consistent with this hypothesis. Thus, a growth factor or activator may be needed for sperm motility and its gene may be missing in XO<u>Sxr</u> mice. However, Eicher <u>et al.</u> (1983) failed to consider an important point. The testicular microenvironment of XO<u>Sxr</u> may be inappropriate, whereas the microenvironment in X^Y O mice may be nearly-normal to normal. Essential factors for sperm motility are produced and secreted by Sertoli cells, and subsequently transposed to the epididymis, where

sperm acquire motility. These factors include metabolites such as pyruvate and lactate (Skinner, 1987) and proteins such as transferrin and androgen binding protein (Griswold, 1988). If the communication between the Sertoli cells and the germ cells is impaired, then these factors may not be released in the correct temporal or spatial manner. Sertoli cell-germ cell communication may not be normal in XO*Sxr* mice. Further, Eicher finds that some $X^{Y}O$ mice are fertile, suggesting that normal, or at least near-normal, numbers of sperm are present in these mice. If normal numbers of sperm are present in $X^{Y}O$ mice then the testicular microenvironment is appropriate for sperm-cell differentiation. This lead to the conclusion that XO*Sxr* and $X^{Y}O$ mice have quite different testicular microenvironments Thus, it is possible that factors necessary for sperm motility may not be available to developing germ cells in XO*Sxr* mice but are available in normal mice. Therefore, it cannot be definitely demonstrated from this evidence that there is a motility gene on the Y chromosome, missing in XO*Sxr* mice, but present in X^{Y} mice. The present studies also suggest that sperm motility *in vitro* is not essential to binding, but certainly increases efficiency of binding to the zona pellucida.

Conclusion

These results demonstrate that all sperm-specific structures and at least two sperm-specific proteins are present and differentiated in the correct temporal and spatial pattern in spermatids of XO*Sxr* mice. Thus, the genes encoding these structures are not located within the

portion of the Y chromosome that XO<u>Sxr</u> mice lack but are possibly within the <u>Sxr</u> segment or, more likely, on the autosomes or X chromosome. Since Sertoli cells are morphologically normal in XO<u>Sxr</u> mice and the genes required for their differentiation are encoded on the Y chromosome, the <u>Sxr</u> fragment must contain one or more of the genes that are necessary for the development of Sertoli cells. Further, these results demonstrated that sex vesicles are present in XO<u>Sxr</u> mice and imply that an intact Y chromosome is not necessary for the elaboration of that structure. Ultrastructural analyses of XO<u>Sxr</u> mice revealed a high frequency of spermatid abnormalities that may have resulted from disturbed cell interactions between the Sertoli and germ cells, from the lack of Y-encoded products missing in XO<u>Sxr</u> mice, or from other indirect effects of the mutation. In addition, significant reductions were observed in the spermatocytes and post-meiotic cells of XO<u>Sxr</u> mice, probably caused by aberrant behavior of the X<u>Sxr</u> chromosome at prophase I, the time when sex-chromosome pairing should occur, and/or by incomplete sex-chromosome inactivation. The dramatic reduction in spermatids of XO<u>Sxr</u> mice was probably a reflection of abnormalities occurring earlier in spermatogenesis combined with inadequate communication between spermatids and Sertoli cells. Finally, these results show that sperm from XO<u>Sxr</u> mice were able to bind to the zonae pellucidae of mouse eggs, but did so at a reduced efficiency. Sperm ligands that are essential for zona binding must, therefore, not be encoded within the portion of the Y chromosome missing in XO<u>Sxr</u> mice. However, sperm of

XO<u>Sxr</u> mice were non-motile, a result suggesting either that Sertoli cell-germ cell communication is probably inappropriate or that there may be a motility factor encoded on the Y chromosome.

In summary, the results suggest that at least one gene encoded on the Y chromosome, within the <u>Sxr</u> segment, is necessary for spermatogenesis: a gene controlling Sertoli cell differentiation. Genes controlling sperm-specific structures are not encoded on the portion of the Y chromosome missing in XO<u>Sxr</u> mice. Abnormalities of X chromosome or <u>Sxr</u> region in inactivation and pairing may be responsible for the reductions, and possibly the abnormalities, observed in spermatocytes and spermatids of XO<u>Sxr</u> mice. Sertoli cell-germ cell communication and fertility may also be impaired because of reduced numbers of germ cells. Therefore, a number of anomalous events may be responsible for spermatogenic abnormalities observed in XO<u>Sxr</u> mice. This study has demonstrated the complexities of spermatogenic control. It has also demonstrated that the major portion of the mouse Y chromosome does not encode products absolutely essential for the assembly of a sperm cell.

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